Slc11a1 limits intracellular growth of *Salmonella enterica* sv. Typhimurium by promoting macrophage immune effector functions and impairing bacterial iron acquisition

Running title:

Slc11a1 limits *Salmonella* iron access

Authors:

Manfred Nairz[1], Gernot Fritsche[1], Marie-Laure V. Crouch[2], Howard C. Barton[3], Ferric C. Fang[2,4], Günter Weiss[1]*

Affiliations:

[1]Department of Internal Medicine I, Clinical Immunology and Infectious Diseases, Innsbruck Medical University, Innsbruck, Austria

Departments of [2]Laboratory Medicine and [4]Microbiology, University of Washington, Seattle, Washington, United States of America

[3]School of Biological Sciences, University of Southampton, Southampton, United Kingdom

[*]For correspondence: Dr. Günter Weiss, Medical University Innsbruck, Department of Internal Medicine I, Clinical Immunology and Infectious Diseases, Anichstr. 35; A-6020 Innsbruck, Austria. Phone: +43-512-504-23255. Fax:+ 43-512-504-25607. E-mail address: guenter.weiss@i-med.ac.at

Abstract

The natural-resistance associated macrophage protein 1, Slc11a1, is a phagolysosomal transporter for protons and divalent ions including iron, that confers host protection against diverse intracellular pathogens including *Salmonella*. We investigated and compared the regulation of iron homeostasis and immune function in RAW264.7 murine phagocytes stably transfected with non-functional Slc11a1 and functional Slc11a1 controls in response to an infection with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). We report that macrophages lacking functional Slc11a1 displayed an increased expression of transferrin receptor 1, resulting in enhanced acquisition of transferrin-bound iron. In contrast, cellular iron release mediated via ferroportin 1 was significantly lower in *Salmonella*-infected Slc11a1-negative macrophages in comparison to phagocytes bearing Slc11a1. Lack of Slc11a1 led to intracellular persistence of *S.* Typhimurium within macrophages which was paralleled by a reduced formation of nitric oxide, tumour necrosis factor-alpha and interleukin-6 in Slc11a1-negative macrophages following *Salmonella* infection, whereas interleukin-10 production was increased. Moreover, Slc11a1-negative phagocytes exhibited higher cellular iron content, resulting in increased iron acquisition by intracellular *Salmonella*. Our observations indicate a bifunctional role for Slc11a1 within phagocytes. Slc11a restricts iron availability, which firstly augments pro-inflammatory macrophage effector functions and secondly concomitantly limits microbial iron access.

Introduction

The natural resistance-associated macrophage protein 1, Slc11a1 (solute carrier family 11 member 1, formerly Nramp1), modifies macrophage activation, thus conferring innate resistance to intracellular pathogens as well as susceptibility to certain autoimmune diseases (Blackwell *et al.*, 1999, Canonne-Hergaux *et al.*, 1999). Slc11a1 is an integral membrane protein expressed in the late phagolysosomes of macrophages and in intracellular compartments of neutrophils and dendritic cells (Gruenheid *et al.*, 1999, Valdez *et al.*, 2008a). Although Slc11a1 primarily acts as a proton-dependent transporter for divalent ions such as iron or manganese, it also exerts pleiotropic effects on innate immune functions. For instance, Slc11a1 modulates the production of chemokines and cytokines such as macrophage inflammatory protein 1-alpha (Mip-1α), tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1ß, and the formation of reactive oxygen and nitrogen species (ROS and RNS, respectively), as well as antigen processing and presentation (Blackwell *et al.*, 2003, Forbes *et al.*, 2001, Kuhn *et al.*, 1999, Nevo *et al.*, 2006, Fritsche *et al.*, 2003, Valdez *et al.*, 2008b).

Slc11a1-mediated resistance to intramacrophage microbes covers distinct taxonomically unrelated pathogens such as *Salmonella*, *Mycobacterium* and *Leishmania* species, and part of this appears to be linked to its capacity to transport iron (Forbes *et al.*, 2001, Huynh *et al.*, 2008). While Slc11a1 may shift iron into phagolysosomes (Zwilling *et al.*, 1999, Kuhn *et al.*, 1999, Kuhn *et al.*, 2001), where redox-active ferrous iron may directly support the formation of ROS in the Fenton reaction, evidence is abundant that Slc11a1 expression is associated with iron efflux out of phagolysosomes and subsequently out of macrophages (Atkinson *et al.*, 1999, Mulero *et al.*, 2002, Fritsche *et al.*, 2007), supporting the hypothesis that Slc11a1 may deprive intracellular microorganisms of essential iron.

Notably, the efficacy of several macrophage immune functions is strongly influenced by iron. Whereas trace amounts of iron are required for the generation of ROS, several pathways of

host protection supported by mononuclear phagocytes, including those driven by interferon-gamma (IFN-γ), are impaired by elevated macrophage iron content (Weiss, 2002). For instance, macrophage iron overload results in reduced expression of TNF-α, inducible nitric oxide synthase (iNOS) and major histocompatibility complex (MHC) class II, thus simultaneously impairing cytokine production, bacterial elimination via RNS and antigen presentation (Weiss *et al.*, 1994, Oexle *et al.*, 2003, Nairz *et al.*, 2007, Recalcati *et al.*, 1998). Importantly, TNF-α signalling and iNOS expression are of pivotal importance in innate immunity against *Salmonella* Typhimurium (Vazquez-Torres *et al.*, 2000, Vazquez-Torres *et al.*, 2001, Mastroeni *et al.*, 2000). Since iron exerts inhibitory effects on these antimicrobial pathways, iron overload of the mononuclear phagocyte system is associated with an increased incidence of infections, as seen in secondary iron overload (Nairz *et al.*, 2006, Rahav *et al.*, 2006, Wang *et al.*, 2003).

Given the importance of iron for innate immune functions and metabolism, macrophages tightly control cellular iron homeostasis by regulating both transmembraneous iron fluxes and intracellular iron storage (Knutson *et al.*, 2003a, Weiss, 2005, Theurl *et al.*, 2005, Oppenheimer, 2001). Phagocytes can acquire non-transferrin-bound iron (NTBI) via divalent metal transporter 1 (Dmt1) and transferrin-bound iron (TBI) via transferrin receptor 1 (TfR1). The phagocytosis of senescent erythrocytes provides macrophages with heme-iron, which is released upon enzymatic degradation by heme oxygenase 1 (Hmox1). Surplus iron can be either stored within ferritin or exported out of macrophages via ferroportin 1 (Fpn1), thus being recycled to the circulating pool of TBI (Bonnah *et al.*, 2004, Nairz *et al.*, 2006, Hentze *et al.*, 2004).

As a consequence of its involvement in central metabolic processes, iron directly promotes the growth and proliferation of microorganisms by serving as an essential nutrient (Weinberg, 1998, Weinberg, 2000, Schaible *et al.*, 2005). In fact, with the sole exception of *Borrelia burgdorferi*, which uses manganese in place of iron-sulfur clusters in its metabolic enzymes,

all mammalian pathogens require iron (Schaible *et al.*, 2004). Accordingly, microbial iron uptake mechanisms promote virulence whereas genetic deletion of iron import pathways reduces pathogenicity (Boyer *et al.*, 2002, Janakiraman *et al.*, 2000, Fischbach *et al.*, 2006, Crouch *et al.*, 2008, Jung *et al.*, 2008, Schrettl *et al.*, 2007, Winkelmann, 2002). The limitation of iron availability for pathogens is thus an effective strategy of innate host defense as shown in several *in vitro* and *in vivo* studies (Ibrahim *et al.*, 2007, Nairz *et al.*, 2007, Olakanmi *et al.*, 2000, Paradkar *et al.*, 2008, Kanti Das *et al.*, 2008).

To better understand the pleiotropic functions of Slc11a1 in host protection against intracellular pathogens, we have initiated *in vitro* experiments to simultaneously study iron homeostasis and effector functions in RAW264.7 macrophage-like cells stably transfected with functional (RAW-37 cells) or non-functional (RAW-21 cells) Slc11a1 during infection with *S.* Typhimurium.

Results

Effects of Slc11a1 on iron acquisition, iron mobilization and iron release by Salmonella-infected macrophages

When investigating macrophage iron import, we found that TfR1 mRNA levels and transferrin-mediated iron uptake were significantly increased in RAW-21 cells lacking functional Slc11a1 when compared with Slc11a1-expressing RAW-37 macrophages. These changes were also seen in *Salmonella*-infected and/or IFN-γ treated cells (Figures 1A and B). In contrast, neither the expression of Dmt1 mRNA nor the uptake of NTBI differed significantly between Slc11a1-non-expressing RAW-21 and Slc11a1-expressing RAW-37 cells (Supplementary figures 1A and B). Moreover, while Fpn1 mRNA levels were not different between solvent-treated RAW-21 and RAW-37 cells, *Salmonella* infection resulted in approximately 80% lower Fpn1 mRNA expression in Slc11a1-dysfunctional RAW-21 cells ($P < 0.05$). This difference was also apparent in *Salmonella*-infected phagocytes subsequently stimulated with IFN-γ (Figure 1C). In parallel with the reduced Fpn1 expression, cellular iron efflux was approximately 95-100% lower in *Salmonella*-infected RAW-21 cells with or without IFN-γ-stimulation, in comparison to Slc11a1-expressing RAW-37 cells subjected to the same treatment (Figure 1D). The observed changes in TfR1 and Fpn1 mRNA levels were similar to their levels of protein expression (Figure 2). Of relevance, levels of hepcidin antimicrobial peptide (Hamp) mRNA were not altered upon *S.* Typhimurium infection and did not differ between RAW-21 and RAW-37 cells (data not shown).

After 24 hours, Hmox1 transcripts were significantly induced in *Salmonella*-infected phagocytes and further enhanced upon addition of IFN-γ. However, Hmox1 mRNA and protein levels were comparable in RAW-21 and RAW-37 cells. Similarly, mRNA levels of the heme-exporting feline leukaemia virus, subgroup C, receptor (Flvcr) were not different between the two cell lines (data not shown).

Effects of Slc11a1 on cellular iron content and on intracellular iron traffic in Salmonella-infected macrophages

To examine the effects of Slc11a1 on iron availability within *Salmonella*-infected macrophages, we first examined the expression of the iron storage protein ferritin. *Salmonella* infection resulted in a marked enhancement of ferritin mRNA and protein expression in both, RAW-21 and RAW-37 cells, in comparison to unstimulated cells. However, Slc11a1 did not influence ferritin expression as no difference was observed between these two cell lines under any of the experimental conditions (Figures 3A and B).

Nevertheless, as ferritin expression at the protein level under inflammatory conditions is influenced by both iron concentrations and inflammatory stimuli, we determined cellular iron content by means of atomic absorption spectrometry. Iron content decreased following *Salmonella* infection in both cells types, but RAW-21 cells were found to have a 43-63% higher iron content than Slc11a1-expressing RAW-37 cells under the same conditions ($P < 0.05$, Figure 3C).

Impact of Slc11a1 on macrophage immune effector functions

To better understand the effects of Slc11a1 on innate immune effector functions, we determined the concentrations of macrophage-derived mediators in culture supernatants of RAW-21 cells and Slc11a1-expressing RAW-37 stimulated with IFN-γ and/or infected with *Salmonella*. Notably, the production of RNS, measured as the oxidation end-product nitrite in cell culture supernatants, was significantly lower in RAW-21 cells than in Slc11a1-expresssing RAW-37 cells (Figure 4A). This may be due to a transcriptional regulation as we found significantly decreased expression of inducible nitric oxide synthase (iNOS) mRNA in *Salmonella*-infected RAW-21 macrophages in comparison to RAW-37 cells (Supplementary figure 2A). In contrast, Slc11a1 had no effect on the transcription of phox-p47 nor the

production of ROS, the latter determined by measurement of total oxidative capacity (TOC) in cell culture supernatants (data not shown and supplementary figure 2B). Intriguingly, the presence of Slc11a1 also correlated with the expression of several macrophage-derived cytokines. Upon infection with *Salmonella* and with or without the addition of IFN-γ, RAW-21 cells lacking functional Slc11a1 secreted significantly lower amounts of TNF-α and IL-6 (Figures 4B and 4C) and substantially higher amounts of IL-10 in comparison to Slc11a1-expressing RAW-37 macrophages treated identically (Figure 4D). In contrast, IL-1ß levels in cell culture supernatants did not differ markedly between the two cell lines (Supplementary figure 2C). Similarly, Slc11a1 did not influence levels of IL-18 or IL-23 mRNA (data not shown). Of interest, RAW-21 also displayed lower mRNA levels of chemokines monocyte chemoattractant protein (Mcp)-1 and Mip-1α, whereas no effect of Slc11a1 was observed with respect to Ccl-5 or Cxcl-10 mRNA expression (data not shown).

Electrophoretic mobility shift assays were performed to determine whether the impaired production of pro-inflammatory mediators by *Salmonella*-infected and IFN-γ stimulated RAW-21 cells could be attributed to specific transcriptional regulators. Slc11a1-non-expressing RAW-21 cells exhibited higher binding affinities of the transcription factor signal transducer and activator of transcription (STAT)-3 in response to *Salmonella* infection and/or cytokine stimulation for 24 hours (Supplementary figure 3), as recently reported for IFN-γ/LPS stimulation (Fritsche *et al.*, 2008). In contrast, activation of interferon regulatory factor (IRF)-1, nuclear factor (NF)-κB and STAT-1 was not affected by the presence of functional Slc11a1 (Supplementary figure 3 and data not shown).

Impact of Slc11a1 on macrophage antimicrobial activity upon IFN-γ stimulation and iron perturbations

We observed increased bacterial survival in Slc11a1-non-expressing RAW-21 cells which became significant by 16 to 24 hours post-infection (Figure 5A), in agreement with previous

observations (Blackwell *et al.*, 2001, Forbes *et al.*, 2001, Fritsche *et al.*, 2008). Stimulation with IFN-γ significantly reduced *Salmonella* survival in both cell types (Figure 5B). TBI supplementation resulted in a dose-dependent increase in *Salmonella* survival within RAW-21 mononuclear phagocytes, while the presence of Slc11a1 in RAW-37 cells efficiently abrogated this *Salmonella* growth-promoting effect of iron supplementation (Figure 6A). In parallel, TBI dose-dependently reduced the generation of nitrite as well as the secretion of TNF-α by *Salmonella*-infected RAW-21 macrophages, which was not observed in Slc11a1-expressing RAW-37 phagocytes (Figures 6B and 6C). Interestingly, TBI supplementation dose-dependently augmented IL-10 expression in infected RAW-21 cells, but the low IL-10 levels seen in Slc11a1-functional RAW-37 cells were not changed by iron supplementation (Figure 6D). NTBI, delivered as ferrous sulfate, exerted similar effects on intracellular *Salmonella* survival and macrophage nitrite and cytokine production to supplementation with TBI (data not shown).

Effects of synthetic Hamp and of an anti-TfR1 antibody on Salmonella-infected RAW-21 and RAW-37 macrophages

We next investigated the effects of synthetic Hamp and of a blocking anti-TfR1 antibody, both of which are expected to modulate macrophage transmembraneous iron fluxes, on effector functions of RAW-21 cells as compared to Slc11a1-functional RAW-37 phagocytes. Interestingly, the addition of Hamp to *Salmonella*-infected macrophages increased the bacterial load by 55-60% in both RAW-21 and RAW-37 cells, in comparison to cells not treated with Hamp (Figure 7A). However, antibody-mediated blockade of TfR1 reduced the number of intracellular bacteria only in RAW-21, but not in Slc11a1-expressing RAW-37 macrophages. Notably, increased concentrations of nitrite were measured in both RAW-21 and RAW-37 macrophages following the addition of anti-TfR1 antibody (Figure 7B). In contrast, the addition of ferrous sulfate and Hamp impaired the generation of nitrite

exclusively in *Salmonella*-infected RAW-21 cells, but not in infected Slc11a1-competent RAW-37 cells. Of note, Hamp resulted in significant inhibition of TNF-α production in both macrophage cell lines, whereas the addition of a blocking anti-TfR1 antibody had no substantial effect on the production of TNF–α in either RAW-21 or RAW-37 cells (Figure 7C).

Limitation of iron availability for intracellular Salmonella in the presence of Slc11a1

To see whether the alterations of transmembrane iron fluxes mediated by functional Slc11a1 may affect iron acquisition by intracellular bacteria, we directly measured the uptake of ^{59}Fe, delivered as either NTBI or TBI, into internalized *Salmonella*. Notably, the incorporation of NTBI and TBI by intramacrophage bacteria was significantly higher in RAW-21 cells in comparison to *Salmonella* residing within RAW-37 cells expressing functional Slc11a1 (Figures 8A and 8B). Of note, IFN-γ was able to further reduce the availability of both iron forms to phagocytosed *Salmonella* independently of Slc11a1.

Effects of bacterial iron acquisition systems on effector functions of RAW-21 and RAW-37 macrophages

The effect of Slc11a1 on the survival of *S.* Typhimurium strains carrying mutations in *entC*, required for siderophore biosynthesis, with or without mutations in the *sit* and *feo* loci, required for siderophore-independent iron acquisition, was determined. Wild-type *S.* Typhimurium exhibited greater intracellular survival than mutant strains lacking siderophore biosynthesis, and this difference was most pronounced in RAW-21 cells (Figure 9A). RAW-21 cells controlled the replication and survival of all *S.* Typhimurium strains less efficiently than did Slc11a1-expressing RAW-37 cells, although significant differences were only observed upon infection with wild-type *Salmonella* (Figure 9A). Interestingly, the intracellular growth of both wild-type and isogenic *entC* mutant *Salmonella* strains was

enhanced following addition of holo-transferrin to infected Slc11a1-dysfunctional RAW-21 macrophages, whereas the *entC sit feo* triple mutant showed no effect. In contrast, RAW-37 cells limited the growth of all bacterial strains whether or not supplemental holo-transferrin was provided.

The production of nitrite and IL-10 was not affected by *entC*, *sit* or *feo* mutations (Figure 9B and supplementary figure 4), but higher TNF-α levels were elicited by *entC* and *entC sit feo* mutants following infection of RAW-21 cells (Figure 9C). Notably, holo-transferrin inhibited nitrite and TNF-α production by infected Slc11a1-deficient RAW-21 phagocytes but did not appear to affect the lower levels produced by RAW-37 cells (Figures 9B and C).

Discussion

Herein, we provide evidence that Slc11a1 exerts multiple functions within *Salmonella*-infected macrophages, simultaneously influencing both, the expression of antimicrobial immune responses and the access of intracellular bacteria to cellular iron. Specifically, the expression of Slc11a1 reduces intracellular iron levels of *Salmonella*-infected macrophages, which is exerted by at least two pathways: first, a reduction in TfR1-mediated iron uptake and second, the induction of Fpn1-mediated iron efflux (Figure 1). These events limit the availability of iron for *Salmonella* residing within phagosomal vacuoles. In addition, due to regulatory effects of iron on IFN-γ-mediated pathways in macrophages, the reduction in intracellular iron concentration promotes the production of TNF-α and IL-6 as well as the expression of iNOS (Oexle *et al.*, 2003, Weiss *et al.*, 1994, Nairz *et al.*, 2008). In parallel, Slc11a1 either directly or via modulation of pro-inflammatory immune effector pathways suppresses the secretion of IL-10 (Fritsche *et al.*, 2008). Accordingly, Slc11a1-mediated resistance against leishmanial parasites, mycobacteria and salmonellae is associated with a T helper cell type 1 (Th1) response, which favours host defense against intracellular microorganisms (Soo *et al.*, 1998, Kramnik *et al.*, 1994, Valdez *et al.*, 2008a, Valdez *et al.*, 2008b). It is tempting to speculate that the increased production of pro-inflammatory cytokines in conjunction with the reduced production of the anti-inflammatory mediator IL-10 may contribute to both the increased resistance to infection and the increased susceptibility to autoimmune disorders that are associated with Slc11a1 in both mice and humans (Blackwell *et al.*, 2003).

Intriguingly, there were differences in the iron-dependent influence of Slc11a1 on the generation of nitrite, TNF-α, IL-6 and IL-10 in *Salmonella*-infected macrophages (Figure 6). , While the addition of TBI dose-dependently inhibited the generation of nitrite and TNF-α exclusively in RAW-21 phagocytes lacking Slc11a1, Slc11a1-expressing RAW-37 cells were

capable of producing high levels of RNS and TNF-α and of secreting even larger amounts of IL-6 upon gradually increasing TBI supplementation. The opposite was true with IL-10 formation. While Slc11a1-positive cells could limit IL-10 secretion in the face of increasing iron levels, *Salmonella*-infected Slc11a1-negative macrophages responded to the exposition to high concentrations of TBI by producing increasing amounts of IL-10. These differential effects were also observed in response to stimulation with IFN-γ and/or LPS (Fritsche *et al.*, 2008), suggesting that they may not indirectly result from the increased *Salmonella* burden within RAW-21 cells. Being aware of the differences in iron handling between Slc11a1-positive and -negative macrophages and of the fact that iron differently affects pro- and anti-inflammatory immune pathways, these observations can thus be traced back to limitation of labile cytoplasmic iron by Slc11a1-expressing cells. Accordingly, we also suggest that Slc11a1-expressing cells can rapidly shift iron from the cytoplasm out of cells via Fpn1. In contrast, iron accumulates in Slc11a1-negative cells and then exerts its negative effects on pro-inflammatory immune effectors pathways while promoting the formation of IL-10 (Fritsche *et al.*, 2008, Knutson *et al.*, 2003b, Mencacci *et al.*, 1997, Oexle *et al.*, 2003, Oppenheimer, 2001, Weiss *et al.*, 1999).

To date, the underlying molecular mechanisms by which Slc11a1 concomitantly increases the production of TNF-α, IL-6 and RNS and differently affects the expression of immune genes have not been elucidated.. Since TNF-α, IL-6, Mcp-1, Mip-1α and iNOS mRNA levels are substantially higher in Slc11a1-expressing RAW-37 cells (Supplementary figure 2 and additional data not shown), a transcriptional or post-transcriptional mechanism may be involved. Notably, *Salmonella*-infected and/or IFN-γ-stimulated RAW-21 and RAW-37 macrophages showed comparable binding activities of the transcription factors IRF-1, NF-κB and STAT-1, all of which are pivotal for the up-regulation of iNOS in response to inflammatory stimuli (Fritsche *et al.*, 2003). However, these differences were only observed at late time-points, whereas differential cytokine mRNA expression was evident earlier.

Therefore, the increased activation of STAT-3 in LPS- or *Salmonella*-stimulated RAW-21 macrophages is presumably a consequence rather than a cause of the higher IL-10 levels produced by these cells. In this context, it is of considerable interest that the neutralization of IL-10 by specific antibodies restored the induction of TNF-α and iNOS and enhanced *Salmonella* killing of *Salmonella* in *Slc11a1*-deficient RAW-21 cells (Fritsche *et al.*, 2008). Some investigators have suggested that Slc11a1 may promote TNF-α and RNS formation by prolonging the half-life of TNF-α and iNOS mRNAs, respectively (Brown *et al.*, 1997), although we were unable to corroborate this observation (Fritsche *et al.*, 2003). Moreover, it is possible that TNF-α produced in greater quantities by Slc11a1-positive macrophages contributes to their higher capacity to generate RNS by acting as a co-activator of iNOS expression (Ables *et al.*, 2001). Thus, Slc11a1 may regulate cytokine expression via activation of iron- or NO-sensitive transcription factors, although we did not detect differential activations of NF-κB and NF-IL6 (Supplementary figure 3 and details not shown). Alternatively, it is possible that functional Slc11a1 may selectively stimulate certain macrophage functions by its ability to promote the maturation of *Salmonella*-containing phagosomes (Cuellar-Mata *et al.*, 2002, Jabado *et al.*, 2003). Intracellular fusion events involving pathogen-containing vesicles may initiate signal transduction mechanisms ensuring a predominately pro-inflammatory response in Slc11a1-expressing infected macrophages. Anyhow, regulatory interactions between iron availability and cellular immune effector pathways are likely to account for some of the effects of Slc11a1 on host resistance to diverse intracellular pathogens such as *Salmonella*, *Mycobacterium* or *Leishmania* spp. (Diefenbach *et al.*, 1999, Mastroeni *et al.*, 2000, Weiss *et al.*, 1994)

Since phagocytes are a preferred habitat for *S.* Typhimurium (Vazquez-Torres *et al.*, 1999), the control of iron homeostasis in infected mononuclear cells is crucial for effective host defense against this bacterium (Collins, 2003, Theurl *et al.*, 2005). Within macrophages, Slc11a1 is found exclusively in the membrane of late endosomes and phagolysosomes but has

not been detected in the plasma membrane (Blackwell *et al.*, 2003, Searle *et al.*, 1998, Atkinson *et al.*, 1997, Gruenheid *et al.*, 1999, Gruenheid *et al.*, 1997, Canonne-Hergaux *et al.*, 2002, Lam-Yuk-Tseung *et al.*, 2006). Therefore, the effect of Slc11a1 on cellular iron release is presumably an indirect one. This may be a consequence of the differences in immune activation between Scl11a1-positive and -negative macrophages, since increased formation of NO and oxygen radicals, as seen in Slc11a1-expressing phagocytes, can directly affect the orchestration of cellular iron homeostasis via iron regulatory proteins (IRPs). Specifically, NO and oxygen radicals modify IRP binding affinity to IREs thereby affecting ferritin and TfR1 mRNA processing (Muckenthaler *et al.*, 2008, Recalcati *et al.*, 1998, Weiss *et al.*, 1993). Our results show reduced acquisition of iron by intraphagolysosomal *Salmonella* residing within Slc11a1-functional cells, which strongly supports the concept that Slc11a1 shifts iron from the phagolysosomal compartment to the cytosolic space. An intermittent cytosolic accumulation of iron may affect Fpn1 and TfR1 expression via both transcriptional and IRP/IRE-mediated posttranscriptional control (Figures 1 and 2). In addition, the presence of Fpn1 in the cell surface membrane can be regulated at the posttranslational level by Hamp produced by activated macrophages in an autocrine fashion (Theurl *et al.*, 2008). However, the cytosolic space does not appear to be the final resting location for this iron, as in steady state conditions, a fall in TfR1-mediated iron uptake along with increased Fpn1-mediated iron export markedly reduced total macrophage iron content and the cytoplasmic labile iron pool, as measured by atomic absorption spectrometry (Figure 4) and electrophoretic mobility shift assays of IRP binding (Atkinson *et al.*, 1999, Barton *et al.*, 1999, Fritsche *et al.*, 2007, Nairz *et al.*, unpublished observations). Slc11a1 did not affect the expression of ferritin or the incorporation of ^{59}Fe into ferritin multimers (Figure 3 and data not shown). Furthermore, we did not detect any effect of Slc11a1 on the expression of the heme-mobilizing enzyme Hmox1 or the heme-exporter Flvcr in infected macrophages. Although we did not challenge macrophages with heme-iron, hemoglobin or erythrocytes, our results support the idea that

Slc11a1 modulates the cytosolic iron content. Iron-sensitive fluorogenic probes targeting specific organelles may be helpful in investigating Slc11a1-mediated iron fluxes between intracellular compartments under real-time conditions by means of microfluorescence imaging (Glickstein *et al.*, 2005, Jabado *et al.*, 2000). However, Slc11a1 leads to reduction of cytoplasmic iron levels and withholds the metal from pathogens residing within phagolysosomes. As Hamp mRNA levels were not affected by the presence or absence of Slc11a1, it is highly unlikely that changes in Hamp expression are responsible for the differences in iron export observed between RAW-21 and Slc11a1-expressing RAW-37 macrophages. Although the blockade of Fpn1-mediated iron efflux by the addition of synthetic Hamp resulted in a marked increase in the microbial burden of infected phagocytes, Hamp treatment of infected macrophages failed to abolish the differences in bacterial survival between RAW-21 and RAW-37 cells, suggesting that Slc11a1-dependent antimicrobial actions are not dependent on Fpn1-mediated iron export (Figure 7).

A number of studies have attempted to elucidate the biochemical function of Slc11a1, yet its mode of action remains a topic of some debate. Experiments with isolated phagosomes suggested that Slc11a1 might transport iron *into* these organelles to provide a catalyst for the production of cytotoxic ROS via the Fenton reaction (Kuhn *et al.*, 1999, Kuhn *et al.*, 2001, Zwilling *et al.*, 1999). However, investigations using intact cells rather indicated that Slc11a1 fluxes divalent ions such as iron and manganese *out of* the lumen of phagosomes into the cytosol (Jabado *et al.*, 2002, Gomes *et al.*, 1998, Forbes *et al.*, 2003). Moreover, the topology of Slc11a1 in the vacuolar membrane relative to the proton gradient indicates that the direction of cation transport is outward. Although we did not directly measure iron transport across the phagolysosomal membrane or iron concentrations within the phagolysosome, our demonstration of reduced iron acquisition by intraphagolysosomal *Salmonella* residing within Slc11a1-expressing cells is most consistent with the idea that Slc11a1 depletes the phagolysosome of iron. To the best of our knowledge, this is the first report to directly

compare ^{59}Fe acquisition by an intraphagolysosomal pathogen in the presence or absence of functional Slc11a1. Studies with *Slc11a1*^{Gly169} (wildtype) and *Slc11a1*^{Asp169} (mutant/functional null) mice and with peritoneal macrophages isolated from these will be of interest to see whether the effect of Slc11a1 is also relevant in primary macrophages and the *in vivo* situation. This apprears likely since a recent study employing *Slc11a1/Nramp1*$1^{+/+}$ and *Slc11a1/Nramp1*$^{-/-}$ mice in a model of *Salmonella*-induced colitis demonstrated that the Slc11a1 phenotype was associated with a pro-inflammatory immune response and an increased clearance of the bacteria (Valdez *et al.*, 2008b).

Of note, it has been demonstrated that *Leishmania amazonensis* is capable of up-regulating its ferrous iron importer LIT1 within phagolysosomes and that this regulation is accelerated in response to iron starvation by functional Slc11a1 (Huynh *et al.*, 2006). Whether or not this also applies to intraphagolysosomal *Salmonella* is currently unknown. However, *Salmonella* expresses a set of virulence genes encoded within *Salmonella* pathogenicity island 2 as a specific response to Slc11a1-mediated iron starvation (Zaharik *et al.*, 2002). In addition, *Mycobacterium tuberculosis* reportedly enhances siderophore production upon Slc11a1-mediated iron deprivation (Jabado *et al.*, 2003).

The divalent cation transporters SitABCD and MntH have been shown to promote *Salmonella* growth in macrophages expressing Slc11a1 (Zaharik *et al.*, 2004). Here we show for the first time that EntC-dependent catecholate-type siderophore production promotes *Salmonella* growth in both Slc11a1-expressing and Slc11a1-deficient macrophages (Figure 8A). The ability of RAW-37 cells to effectively restrict wild type *S.* Typhimurium growth in phagocytes illustrates the capacity of Slc11a1 to limit the availability of cations in the vacuolar compartment beyond even the ability of high-affinity microbial iron acquisition systems to obtain them.

Since iron plays a central role in the interaction between host and pathogen, affecting both microbial replication and the activation of cell-mediated immune pathways, a better

understanding of Slc11a1 transporter function and the further elucidation of the mechanisms linking metal ion redistribution to pro- and anti-inflammatory gene expression might enable the development of pharmacological strategies to specifically target Slc11a1 as a novel approach to control infections with intracellular pathogens such as *Salmonella* and *Mycobacterium tuberculosis*.

We have provided evidence to support a dual role of the phagolysosomal transport protein Slc11a1 in promoting host resistance against intracellular microbes such as *S.* Typhimurium. By reducing the intracellular iron content of infected macrophages, Slc11a1 starves ingested bacteria of iron while concomitantly promoting the generation of antimicrobial effector molecules. These properties of Slc11a1 provide important insights into mechanisms by which nutrient withholding contributes to innate immunity.

Experimental procedures

Cell culture

RAW264.7 murine macrophage-like cells were originally isolated from Slc11a1-non-functional BALB/c mice and obtained from the American Type Culture Collection. RAW264.7 cells were stably transfected by standard methods using the pHßA-1-*neo* expression plasmid containing the full-length *Slc11a1* cDNA (RAW-37) or an antisense-*Slc11a1* construct (RAW-21) as described previously (Atkinson *et al.*, 1999). Routinely, cells were grown in DMEM (containing 0.1 mg/ml $Fe(NO_3)_3 \cdot 9H_2O$; obtained from Biochrom, Berlin, Germany) containing 10% FCS, 2 mM L-glutamine and 50 µg/ml ampicillin at 37°C in humidified air containing 5% CO_2 and periodically selected for plasmid retention using G418 (Invitrogen). Stable expression of Slc11a1 in RAW-37 phagocytes was confirmed by immunoblotting (Supplementary figure 5, upper panel).

For infection experiments, $1x10^6$ RAW-21/37 cells were seeded into 6-well plates in 2 ml of DMEM containing 10% FCS, 2 mM L-glutamine and 50 µg/ml ampicillin. *S.* Typhimurium strain C5RP4 (kindly provided by Dr. Pietro Mastroeni, Cambridge University, Cambridge, United Kingdom), a virulent wild-type isolate carrying natural resistance to ampicillin, was used for experiments unless otherwise indicated and grown in Luria-Bertani (LB) broth (obtained from Sigma) containing 50 µg/ml ampicillin to late-logarithmic phase. After preincubation of *S.* Typhimurium in complete DMEM at 37°C for 20 minutes, RAW-21/37 cells were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 10 for 1 hour exactly as described (Nairz *et al.*, 2007). Control cells were treated with complete DMEM without any microbes. After 1 hour, RAW-21/37 cells were washed three times with phosphate-buffered saline (PBS; purchased from Invitrogen) and incubated in 2 ml of complete DMEM additionally containing 20 µg/ml of gentamicin (Gibco) in order to kill extracellular bacteria. Thereafter, macrophages were stimulated with recombinant murine

IFN-γ (50 U/ml; obtained from R&D), whereas controls were treated with PBS as solvent. After incubation for an additional 23 hours, RAW-21/37 cells were washed three times in PBS and subjected to RNA or protein isolation. Alternatively, cells were treated with lipopolysaccharide (LPS; obtained from Sigma) and IFN-γ and subjected to RNA isolation.

For certain experiments, RAW-21/37 cells were cultured in complete DMEM without any antibiotics and infected with *S.* Typhimurium strain MLC446 (wild-type; corresponding to ATCC 14028s) or isogenic mutant derivatives MLC619 (*entC*::aph; single mutant) and MLC774 (*entC*::*aph sit*::*bla feo*::*Tn*10 (Tet^r); triple mutant). Mutant strains were constructed and grown as described (Crouch *et al.*, 2008) and used as detailed above.

For quantification of intracellular bacteria by means of gentamicin protection, RAW-21/37 cells were infected as described above and stimulated with IFN-γ, serial concentrations of human holo-transferrin or ferrous sulfate (all from Sigma). In experiments using synthetic murine hepcidin 1 (1 μM; purchased from PeptaNova), a blocking monoclonal anti-TfR1 antibody and desferrioxamine (DFO; added at a concentration of 50 μM and purchased from Sigma), cells were pre-treated for 8 hours and subsequently infected for an additional 12 hours. The appropriate solvent or isotype control antibody served as controls. After an infection period of 24 hours, cells were washed 5 times with PBS, lysed in 0.5% deoxycholic acid (Sigma) and plated under sterile conditions in appropriate dilutions onto LB agar plates.

Quantitative Reverse Transcription PCR (qPCR)

Preparation of total RNA from RAW-21/37 macrophages was performed by a guanidinium-isothiocyanate-phenol-chloroform based extraction method exactly as described (Ludwiczek *et al.*, 2007). Quantification of mRNA expression was carried out by means of quantitative reverse transcription PCR (qPCR), which was performed exactly as described (Ludwiczek *et al.*, 2004). Amplifications conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for

15 s, 60°C for 60 s. The following primers and TaqMan probes (Microsynth), the latter carrying 5'-FAM and 3'-BHQ1-labels, were used:

mu-Dmt1: 5'-CCAGCCAGTAAGTTCAAGGATCC-3', 5'-GCGTAGCAGCTGATCTGGG-3', 5'-TGGCCTCGCGCCCCAACA-3',

mu-TfR1: 5'-CGCTTTGGGTGCTGGTG-3', 5-GGGCAAGTTTCAACAGAAGACC-3', 5'-CCCACACTGGACTTCGCCGCA-3',

mu-Fpn1: 5'-CTACCATTAGAAGGATTGACCAGCT-3', 5'-CAAATGTCATAATCTGGCCGA-3', 5'-CAACATCCTGGCCCCCATGGC-3',

mu-Hamp: 5'-TGTCTCCTGCTTCTCCTCCTTG-3', 5'-AGCTCTGTAGTCTGTCTCATCTGTTG-3', 5'-CAGCCTGAGCAGCACCACCTATCTCC-3',

mu-Hmox1: 5'-GTGATGGAGCGTCCACAGC-3', 5'-TGGTGGCCTCCTTCAAGG-3', 5'-CGACAGCATGCCCCAGGATTTGTC-3',

mu-H-ferritin: 5'-GCGAGGTGGCCGAATCT-3', 5'-CAGCCCGCTCTCCCAGT-3', 5'-CCTGCAGGATATAAAGAAACCAGACCGTGA-3',

mu-Flvcr: 5'-ATCTGGAACCTGTGCAGAAACA-3', 5'-ATTGAATAAAATGCTCCAGTCATGAT-3', 5'-CCCCTTTGTTCTCCTGCTGGTCAGTTATG-3',

mu-iNOS: 5'-CAGCTGGGCTGTACAAACCTT-3', 5'-CATTGGAAGTGAAGCGTTTCG-3', 5'-CGGGCAGCCTGTGAGACCTTTGA-3',

mu-phox-p47: 5'-GAGGCGGAGGATCCGG-3', 5'-TCTTCAACAGCAGCGTACGC-3', 5'-CAACTACGCAGGTGAACCGTATGTAACCATCA-3',

mu-TNF-α: 5'- TTCTATGGCCCAGACCCTCA-3', 5'-TTGCTACGACGTGGGCTACA-3', 5'- CTCAGATCATCTTCTCAAAATTCGAGTGACAAGC-3',

mu-IL-6: 5'- TGTTCTCTGGGAAATCGTGGA-3', 5'-AAGTGCATCATCGTTGTTCATACA-3', 5'-ATGAGAAAAGAGTTGTGCAATGGCAATTCTG-3',

mu-IL-10: 5'- CCAGAGCCACATGCTCCTAGA-3', 5'-TGGTCCTTTGTTTGAAAGAAAGTCT-3', 5'- TGCGGACTGCCTTCAGCCAGG-3',

mu-IL-18: 5'-GACTCTTGCGTCAACTTCAAGGA-3', 5'-TTGTCTGATTCCAGGTCTCCATT-3', 5'-TGATGTTTATTGACAACACGCTTTACTTTATACCTGAAGA-3',

mu-IL-23-p19: 5'-AGCGGGACATATGAATCTACTAAGAGA-3', 5'-GTCCTAGTAGGGAGGTGTGAAGTTG-3', 5'- CCAGTTCTGCTTGCAAAGGATCCGC-3'

mu-IL-23-p40: 5'-GACCATCACTGTCAAAGAGTTTCTAGAT-3', 5'-AGGAAAGTCTTGTTTTTGAAATTTTTTAA-3', 5'-CCACTCACATCTGCTGCTCCACAAGAAG-3',

mu-Mcp-1: 5'-CTTCTGGGCCTGCTGTTCA-3', 5'-CCAGCCTACTCATTGGGATCA-3', 5'-CTCAGCCAGATGCAGTTAACGCCCC-3',

mu-Mip-1α: 5'-AGCTGACACCCCGACTGC-3', 5'-GTCAACGATGAATTGGCGTG-3', 5'-TGCTGCTTCTCCTACAGCCGGAAGAT-3',

mu-18S rRNA: 5'-CCTGCCCTTTGTACACACCG-3', 5'-CGATCCGAGGGCCTCAC-3', 5'-CCGTCGCTACTACCGATTGGATGGTTT-3'.

Unless otherwise indicated, qPCR data are expressed as mean ± SD of five independent experiments.

Immunoblot analysis

Protein extracts from RAW-21/37 cells were prepared using the radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50

mM Tris [tris(hydroxymethyl)aminomethane] HCl, pH 8.0;) supplemented with 1 μg/ml aprotinin and 1 μg/ml leupeptin (all from Sigma). 10 - 40 μg of total protein were run on 8 - 15% SDS-polyacrylamide gels and immunoblotting was performed exactly as described (Theurl *et al.*, 2006) using a mouse anti-human TfR1 antibody (1:1000; Zymed), a rabbit anti-human ferritin antibody (1:800; Dako), a rabbit anti-human ferroportin 1 antibody (1:400; Eurogentec), a rabbit anti-Slc11a1/Nramp1 antibody (1:1000; (Atkinson *et al.*, 1999)), a rabbit anti-rat Hmox1 (1:2000; Stressgen) or a rabbit anti-Actin antibody (1:1000; Sigma), the latter used as a loading control.

Quantification of iron uptake and release by macrophages

For macrophage iron uptake and release studies, RAW-21/37 cells were infected with *S.* Typhimurium as detailed above. Following three washing cycles with serum-free, HEPES-buffered DMEM (2 mM L-glutamine, 50 μg/ml ampicillin, 25 mM HEPES, pH 7.4), cells were then incubated therein. For determination of the uptake of non-transferrin-bound iron (NTBI), 5 μM ^{59}Fe-citrate (DuPont New England Nuclear) were used, whereas for the measurement of transferrin-bound iron (TBI) acquisition, ^{59}Fe-labeled transferrin was added at a concentration of 12.5 μg/ml exactly as described (Ludwiczek *et al.*, 2003). For iron release experiments, cells were first incubated with 5 μM ^{59}Fe-citrate for 4 hours to allow for iron loading and then washed four times and repleted with 2 ml of serum-free, HEPES-buffered DMEM. After an additional incubation for 2 hours, cellular iron release was measured by means of a γ-counter as described (Nairz *et al.*, 2008). In parallel to each iron release study, a trypan-blue exclusion assay was performed to ensure that neither treatment interfered with the integrity of the RAW-21/37 cell surface membrane.

Determination of iron acquisition by S. Typhimurium

RAW-21/37 cells were seeded in complete DMEM and infected with *S.* Typhimurium strain C5RP4 at an MOI of 10 as detailed above. Thereafter, cells were washed three times and repleted with serum-free, HEPES-buffered DMEM. After the addition of either 5 μM ^{59}Fe-citrate or 12.5 μg/ml of ^{59}Fe-labeled transferrin, macrophages were incubated for additional 4 hours. Thereafter, intracellular bacilli were harvested according to a modified protocol as described (Olakanmi *et al.*, 2002). Briefly, RAW-21/37 cells were lysed in 0.1% SDS in the presence of 10,000 U/ml DNase-I (Invitrogen) and EDTA-free protease inhibitor (Roche). Cell lysates were withdrawn into microfuge tubes and incubated at room temperature for 10 min, and aliquots of 50 μl volume were transferred into γ-counter tubes for assessment of total iron in the macrophage lysate. The released bacilli were centrifuged at 10,000 *g* for 10 min at room temperature. The supernatant was removed, and the bacterial pellet was washed three times with 0.01% SDS in DMEM containing 1 mg/ml proteinase K (Roche). The bacilli were finally resuspended in 500 μl of 0.01% SDS in DMEM containing 1 mg/ml proteinase K. Fifty μl of the bacterial suspension were plated in serial dilutions onto agar plates to quantify released bacteria while the remaining volume was filtered through centrifugal filter devices with a PDVF membrane of 0.22 μm pore size (Millipore). The filters containing the trapped bacilli were washed five times and placed into a γ-counter tube. The amount of *Salmonella*-associated ^{59}Fe was assessed using a γ-counter. No association of ^{59}Fe to *S.* Typhimurium, heat-inactivated at 70°C for 20 min, could be detected. The incorporation of ^{59}Fe into ferritin multimers was investigated exactly as described REF.

Detection of cytokines, ferritin content, nitrite and total oxidative capacity

Concentrations of TNF, IL-1ß, IL-6 and IL-10 in cell culture supernatants were determined by specific ELISA kits (BD Pharmingen). Ferritin content in cytoplasmatic extracts was measured by a specific ELISA kit (Kamiya Biomedical Company) and normalized for total cytoplasmatic protein content as determined by the BCA method (Pierce). Determination of

nitrite, an oxidation product of NO, was carried out to monitor the production of RNS using the Griess reagent (Merck) as described (Fritsche *et al.*, 2001). The formation of ROS was studied by measuring total oxidative capacity (TOC) in culture supernatants with the TOC Kit (Tatzber KG), which is based on the peroxidase-mediated oxidation of tetramethylbenzidine (Weiss *et al.*, 2003).

Electrophoretic mobility shift assays (EMSA)

Extraction of nuclear proteins was carried out as described (Schreiber *et al.*, 1989). For the preparation of radiolabeled probes representing standard consensus sequences of various transcription factors known to mediate the response of murine macrophages to LPS/IFN-γ stimulation, the oligonucleotides used were as follows: Interferon regulatory factor 1 (IRF-1) sense 5'-GGAAGCGAAAATGAAATTG-3', IRF-1 antisense 5'-TGAGTCAATTTCATTTTCG-3';
NF-κB sense 5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3', NF-κB antisense 5'-TCGACCTCTCGGAAAGTCCCCTCTGA-3'; STAT-1 sense 5'-CATGTTATGCATATTCCTGTAAGTG-3', STAT-1 antisense 5'-CGTGCACTTACAGGAATATGCATA-3'; STAT-3 sense 5'-GATCCTTCTGGGAATTCCTA-3', STAT-3 antisense 5'-GATCTAGGAATTCCCAGAAG-3'. For the preparation of double-stranded probes, oligomers were annealed, and overhanging ends were filled with [α-32P]dCTP (Amersham) and the three other non-radiolabeled dNTPs (Pharmacia) using Klenow enzyme (Amersham) as described (Fritsche *et al.*, 2003). For the preparation of unlabeled competitors, dCTP was used. A total of 10 μg of nuclear extracts were preincubated with 2 μg of double-stranded poly(dI-dC)·poly(dIdC) (Pharmacia) on ice for 10 min before addition of 2 ng of the radiolabeled oligonucleotide probe (50,000 cpm/ng). For competition studies, a 30-fold excess of unlabeled oligonucleotide probe was added to the nuclear extracts derived from RAW-21 cells (stimulated with *S.* Typhimurium and IFN-γ for

24 hours) 10 min before addition of the radioactive probe. The DNA binding reactions were performed in the presence of 200 mM HEPES (pH 7.8), 10 mM EDTA, and 10 mM DDT for 20 min on ice. Samples were separated on 6% nondenaturing polyacrylamide gels, which were subsequently exposed by means of autoradiography.

Statistical analysis

Statistical analysis was carried out using a SPSS statistical package. Calculations for statistical differences were carried out by parametric ANOVA test using Bonferroni correction for multiple tests or, where appropriate, by nonparametric Kruskal-Wallis test. Unless otherwise indicated, statistically significant differences between means are indicated as follows: * $P < 0.05$ for infected/stimulated RAW-21 macrophages in comparison to RAW-21 controls; ° $P < 0.05$ for infected/stimulated RAW-37 in comparison to untreated RAW-37 cells; # $P < 0.05$ for the comparison of RAW-21 and RAW-37 cells subjected to the same treatment.

References

Ables, G.P., Takamatsu, D., Noma, H., El-Shazly, S., Jin, H.K., Taniguchi, T.*, et al.* (2001). The roles of Nramp1 and Tnfa genes in nitric oxide production and their effect on the growth of Salmonella typhimurium in macrophages from Nramp1 congenic and tumor necrosis factor-alpha-/- mice. *J Interferon Cytokine Res* **21,** 53-62.

Atkinson, P.G. and Barton, C.H. (1999). High level expression of Nramp1G169 in RAW264.7 cell transfectants: analysis of intracellular iron transport. *Immunology* **96,** 656-662.

Atkinson, P.G., Blackwell, J.M. and Barton, C.H. (1997). Nramp1 locus encodes a 65 kDa interferon-gamma-inducible protein in murine macrophages. *Biochem J* **325 (Pt 3),** 779-786.

Barton, C.H., Biggs, T.E., Baker, S.T., Bowen, H. and Atkinson, P.G. (1999). Nramp1: a link between intracellular iron transport and innate resistance to intracellular pathogens. *J Leukoc Biol* **66,** 757-762.

Blackwell, J.M., Goswami, T., Evans, C.A., Sibthorpe, D., Papo, N., White, J.K.*, et al.* (2001). SLC11A1 (formerly NRAMP1) and disease resistance. *Cell Microbiol* **3,** 773-784.

Blackwell, J.M. and Searle, S. (1999). Genetic regulation of macrophage activation: understanding the function of Nramp1 (=Ity/Lsh/Bcg). *Immunol Lett* **65,** 73-80.

Blackwell, J.M., Searle, S., Mohamed, H. and White, J.K. (2003). Divalent cation transport and susceptibility to infectious and autoimmune disease: continuation of the Ity/Lsh/Bcg/Nramp1/Slc11a1 gene story. *Immunol Lett* **85,** 197-203.

Bonnah, R.A., Muckenthaler, M.U., Carlson, H., Minana, B., Enns, C.A., Hentze, M.W. and So, M. (2004). Expression of epithelial cell iron-related genes upon infection by Neisseria meningitidis. *Cell Microbiol* **6,** 473-484.

Boyer, E., Bergevin, I., Malo, D., Gros, P. and Cellier, M.F. (2002). Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. *Infect Immun* **70,** 6032-6042.

Brown, D.H., Lafuse, W.P. and Zwilling, B.S. (1997). Stabilized expression of mRNA is associated with mycobacterial resistance controlled by Nramp1. *Infect Immun* **65,** 597-603.

Canonne-Hergaux, F., Calafat, J., Richer, E., Cellier, M., Grinstein, S., Borregaard, N. and Gros, P. (2002). Expression and subcellular localization of NRAMP1 in human neutrophil granules. *Blood* **100,** 268-275.

Canonne-Hergaux, F., Gruenheid, S., Govoni, G. and Gros, P. (1999). The Nramp1 protein and its role in resistance to infection and macrophage function. *Proc Assoc Am Physicians* **111,** 283-289.

Collins, H.L. (2003). The role of iron in infections with intracellular bacteria. *Immunol Lett* **85,** 193-195.

Crouch, M.L., Castor, M., Karlinsey, J.E., Kalhorn, T. and Fang, F.C. (2008). Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of Salmonella enterica serovar Typhimurium. *Mol Microbiol* **67,** 971-983.

Cuellar-Mata, P., Jabado, N., Liu, J., Furuya, W., Finlay, B.B., Gros, P. and Grinstein, S. (2002). Nramp1 modifies the fusion of Salmonella typhimurium-containing vacuoles with cellular endomembranes in macrophages. *J Biol Chem* **277,** 2258-2265.

Diefenbach, A., Schindler, H., Rollinghoff, M., Yokoyama, W.M. and Bogdan, C. (1999). Requirement for type 2 NO synthase for IL-12 signaling in innate immunity. *Science* **284,** 951-955.

Fischbach, M.A., Lin, H., Zhou, L., Yu, Y., Abergel, R.J., Liu, D.R.*, et al.* (2006). The pathogen-associated iroA gene cluster mediates bacterial evasion of lipocalin 2. *Proc Natl Acad Sci U S A* **103,** 16502-16507.

Forbes, J.R. and Gros, P. (2001). Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol* **9,** 397-403.

Forbes, J.R. and Gros, P. (2003). Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane. *Blood* **102,** 1884-1892.

Fritsche, G., Dlaska, M., Barton, H., Theurl, I., Garimorth, K. and Weiss, G. (2003). Nramp1 functionality increases inducible nitric oxide synthase transcription via stimulation of IFN regulatory factor 1 expression. *J Immunol* **171,** 1994-1998.

Fritsche, G., Larcher, C., Schennach, H. and Weiss, G. (2001). Regulatory interactions between iron and nitric oxide metabolism for immune defense against Plasmodium falciparum infection. *J Infect Dis* **183,** 1388-1394.

Fritsche, G., Nairz, M., Theurl, I., Mair, S., Bellmann-Weiler, R., Barton, H.C. and Weiss, G. (2007). Modulation of macrophage iron transport by Nramp1 (Slc11a1). *Immunobiology* **212,** 751-757.

Fritsche, G., Nairz, M., Werner, E.R., Barton, H.C. and Weiss, G. (2008). Nramp1-functionality increases iNOS expression via repression of IL-10 formation. *Eur J Immunol* **38,** 3060-3067.

Glickstein, H., El, R.B., Shvartsman, M. and Cabantchik, Z.I. (2005). Intracellular labile iron pools as direct targets of iron chelators: a fluorescence study of chelator action in living cells. *Blood* **106,** 3242-3250.

Gomes, M.S. and Appelberg, R. (1998). Evidence for a link between iron metabolism and Nramp1 gene function in innate resistance against Mycobacterium avium. *Immunology* **95,** 165-168.

Gruenheid, S., Canonne-Hergaux, F., Gauthier, S., Hackam, D.J., Grinstein, S. and Gros, P. (1999). The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. *J Exp Med* **189,** 831-841.

Gruenheid, S., Pinner, E., Desjardins, M. and Gros, P. (1997). Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. *J Exp Med* **185,** 717-730.

Hentze, M.W., Muckenthaler, M.U. and Andrews, N.C. (2004). Balancing acts: molecular control of mammalian iron metabolism. *Cell* **117,** 285-297.

Huynh, C. and Andrews, N.W. (2008). Iron acquisition within host cells and the pathogenicity of Leishmania. *Cell Microbiol* **10,** 293-300.

Huynh, C., Sacks, D.L. and Andrews, N.W. (2006). A Leishmania amazonensis ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. *J Exp Med* **203,** 2363-2375.

Ibrahim, A.S., Gebermariam, T., Fu, Y., Lin, L., Husseiny, M.I., French, S.W.*, et al.* (2007). The iron chelator deferasirox protects mice from mucormycosis through iron starvation. *J Clin Invest* **117,** 2649-2657.

Jabado, N., Canonne-Hergaux, F., Gruenheid, S., Picard, V. and Gros, P. (2002). Iron transporter Nramp2/DMT-1 is associated with the membrane of phagosomes in macrophages and Sertoli cells. *Blood* **100,** 2617-2622.

Jabado, N., Cuellar-Mata, P., Grinstein, S. and Gros, P. (2003). Iron chelators modulate the fusogenic properties of Salmonella-containing phagosomes. *Proc Natl Acad Sci U S A* **100,** 6127-6132.

Jabado, N., Jankowski, A., Dougaparsad, S., Picard, V., Grinstein, S. and Gros, P. (2000). Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane. *J Exp Med* **192,** 1237-1248.

Janakiraman, A. and Slauch, J.M. (2000). The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of Salmonella typhimurium. *Mol Microbiol* **35,** 1146-1155.

Jung, W.H., Sham, A., Lian, T., Singh, A., Kosman, D.J. and Kronstad, J.W. (2008). Iron source preference and regulation of iron uptake in Cryptococcus neoformans. *PLoS Pathog* **4,** e45.

Kanti Das, N., Biswas, S., Solanki, S. and Mukhopadhyay, C.K. (2008). Leishmania donovani depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. *Cell Microbiol*.

Knutson, M. and Wessling-Resnick, M. (2003a). Iron metabolism in the reticuloendothelial system. *Crit Rev Biochem Mol Biol* **38,** 61-88.

Knutson, M.D., Vafa, M.R., Haile, D.J. and Wessling-Resnick, M. (2003b). Iron loading and erythrophagocytosis increase ferroportin 1 (FPN1) expression in J774 macrophages. *Blood* **102,** 4191-4197.

Kramnik, I., Radzioch, D. and Skamene, E. (1994). T-helper 1-like subset selection in Mycobacterium bovis bacillus Calmette-Guerin-infected resistant and susceptible mice. *Immunology* **81,** 618-625.

Kuhn, D.E., Baker, B.D., Lafuse, W.P. and Zwilling, B.S. (1999). Differential iron transport into phagosomes isolated from the RAW264.7 macrophage cell lines transfected with Nramp1Gly169 or Nramp1Asp169. *J Leukoc Biol* **66,** 113-119.

Kuhn, D.E., Lafuse, W.P. and Zwilling, B.S. (2001). Iron transport into mycobacterium avium-containing phagosomes from an Nramp1(Gly169)-transfected RAW264.7 macrophage cell line. *J Leukoc Biol* **69,** 43-49.

Lam-Yuk-Tseung, S., Picard, V. and Gros, P. (2006). Identification of a tyrosine-based motif (YGSI) in the amino terminus of Nramp1 (Slc11a1) that is important for lysosomal targeting. *J Biol Chem* **281,** 31677-31688.

Ludwiczek, S., Aigner, E., Theurl, I. and Weiss, G. (2003). Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood* **101,** 4148-4154.

Ludwiczek, S., Theurl, I., Artner-Dworzak, E., Chorney, M. and Weiss, G. (2004). Duodenal HFE expression and hepcidin levels determine body iron homeostasis: modulation by genetic diversity and dietary iron availability. *J Mol Med* **82,** 373-382.

Ludwiczek, S., Theurl, I., Muckenthaler, M.U., Jakab, M., Mair, S.M., Theurl, M.*, et al.* (2007). Ca2+ channel blockers reverse iron overload by a new mechanism via divalent metal transporter-1. *Nat Med* **13,** 448-454.

Mastroeni, P., Vazquez-Torres, A., Fang, F.C., Xu, Y., Khan, S., Hormaeche, C.E. and Dougan, G. (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J Exp Med* **192,** 237-248.

Mencacci, A., Cenci, E., Boelaert, J.R., Bucci, P., Mosci, P., Fe d'Ostiani, C.*, et al.* (1997). Iron overload alters innate and T helper cell responses to Candida albicans in mice. *J Infect Dis* **175,** 1467-1476.

Muckenthaler, M.U., Galy, B. and Hentze, M.W. (2008). Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. *Annu Rev Nutr* **28,** 197-213.

Mulero, V., Searle, S., Blackwell, J.M. and Brock, J.H. (2002). Solute carrier 11a1 (Slc11a1; formerly Nramp1) regulates metabolism and release of iron acquired by phagocytic, but not transferrin-receptor-mediated, iron uptake. *Biochem J* **363,** 89-94.

Nairz, M., Fritsche, G., Brunner, P., Talasz, H., Hantke, K. and Weiss, G. (2008). Interferon-gamma limits the availability of iron for intramacrophage Salmonella typhimurium. *Eur J Immunol* **38,** 1923-1936.

Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S.M., Fritsche, G. and Weiss, G. (2007). The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular Salmonella typhimurium. *Cell Microbiol* **9,** 2126-2140.

Nairz, M. and Weiss, G. (2006). Molecular and clinical aspects of iron homeostasis: From anemia to hemochromatosis. *Wien Klin Wochenschr* **118,** 442-462.

Nairz, M. and Weiss, G. (unpublished observations).

Nevo, Y. and Nelson, N. (2006). The NRAMP family of metal-ion transporters. *Biochim Biophys Acta* **1763,** 609-620.

Oexle, H., Kaser, A., Most, J., Bellmann-Weiler, R., Werner, E.R., Werner-Felmayer, G. and Weiss, G. (2003). Pathways for the regulation of interferon-gamma-inducible genes by iron in human monocytic cells. *J Leukoc Biol* **74,** 287-294.

Olakanmi, O., Britigan, B.E. and Schlesinger, L.S. (2000). Gallium disrupts iron metabolism of mycobacteria residing within human macrophages. *Infect Immun* **68,** 5619-5627.

Olakanmi, O., Schlesinger, L.S., Ahmed, A. and Britigan, B.E. (2002). Intraphagosomal Mycobacterium tuberculosis acquires iron from both extracellular transferrin and intracellular iron pools. Impact of interferon-gamma and hemochromatosis. *J Biol Chem* **277,** 49727-49734.

Oppenheimer, S.J. (2001). Iron and its relation to immunity and infectious disease. *J Nutr* **131,** 616S-633S; discussion 633S-635S.

Paradkar, P.N., De Domenico, I., Durchfort, N., Zohn, I., Kaplan, J. and Ward, D.M. (2008). Iron depletion limits intracellular bacterial growth in macrophages. *Blood* **112,** 866-874.

Rahav, G., Volach, V., Shapiro, M., Rund, D., Rachmilewitz, E.A. and Goldfarb, A. (2006). Severe infections in thalassaemic patients: prevalence and predisposing factors. *Br J Haematol* **133,** 667-674.

Recalcati, S., Taramelli, D., Conte, D. and Cairo, G. (1998). Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. *Blood* **91,** 1059-1066.

Schaible, U.E. and Kaufmann, S.H. (2004). Iron and microbial infection. *Nat Rev Microbiol* **2,** 946-953.

Schaible, U.E. and Kaufmann, S.H. (2005). A nutritive view on the host-pathogen interplay. *Trends Microbiol* **13,** 373-380.

Schreiber, E., Matthias, P., Muller, M.M. and Schaffner, W. (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17,** 6419.

Schrettl, M., Bignell, E., Kragl, C., Sabiha, Y., Loss, O., Eisendle, M.*, et al.* (2007). Distinct roles for intra- and extracellular siderophores during Aspergillus fumigatus infection. *PLoS Pathog* **3,** 1195-1207.

Searle, S., Bright, N.A., Roach, T.I., Atkinson, P.G., Barton, C.H., Meloen, R.H. and Blackwell, J.M. (1998). Localisation of Nramp1 in macrophages: modulation with activation and infection. *J Cell Sci* **111 (Pt 19),** 2855-2866.

Soo, S.S., Villarreal-Ramos, B., Anjam Khan, C.M., Hormaeche, C.E. and Blackwell, J.M. (1998). Genetic control of immune response to recombinant antigens carried by an attenuated Salmonella typhimurium vaccine strain: Nramp1 influences T-helper subset responses and protection against leishmanial challenge. *Infect Immun* **66,** 1910-1917.

Theurl, I., Fritsche, G., Ludwiczek, S., Garimorth, K., Bellmann-Weiler, R. and Weiss, G. (2005). The macrophage: a cellular factory at the interphase between iron and immunity for the control of infections. *Biometals* **18,** 359-367.

Theurl, I., Mattle, V., Seifert, M., Mariani, M., Marth, C. and Weiss, G. (2006). Dysregulated monocyte iron homeostasis and erythropoietin formation in patients with anemia of chronic disease. *Blood* **107,** 4142-4148.

Theurl, I., Theurl, M., Seifert, M., Mair, S., Nairz, M., Rumpold, H.*, et al.* (2008). Autocrine formation of hepcidin induces iron retention in human monocytes. *Blood* **111,** 2392-2399.

Valdez, Y., Diehl, G.E., Vallance, B.A., Grassl, G.A., Guttman, J.A., Brown, N.F.*, et al.* (2008a). Nramp1 expression by dendritic cells modulates inflammatory responses during Salmonella Typhimurium infection. *Cell Microbiol* **10,** 1646-1661.

Valdez, Y., Grassl, G.A., Guttman, J.A., Coburn, B., Gros, P., Vallance, B.A. and Brett Finlay, B. (2008b). Nramp1 drives an accelerated inflammatory response during Salmonella-induced colitis in mice. *Cell Microbiol*.

Vazquez-Torres, A., Fantuzzi, G., Edwards, C.K., 3rd, Dinarello, C.A. and Fang, F.C. (2001). Defective localization of the NADPH phagocyte oxidase to Salmonella-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. *Proc Natl Acad Sci U S A* **98,** 2561-2565.

Vazquez-Torres, A., Jones-Carson, J., Baumler, A.J., Falkow, S., Valdivia, R., Brown, W.*, et al.* (1999). Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. *Nature* **401,** 804-808.

Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. and Fang, F.C. (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med* **192,** 227-236.

Wang, S.C., Lin, K.H., Chern, J.P., Lu, M.Y., Jou, S.T., Lin, D.T. and Lin, K.S. (2003). Severe bacterial infection in transfusion-dependent patients with thalassemia major. *Clin Infect Dis* **37,** 984-988.

Weinberg, E.D. (1998). Patho-ecological implications of microbial acquisition of host iron. *Reviews in Medical Microbiology* **9,** 171-178.

Weinberg, E.D. (2000). Modulation of intramacrophage iron metabolism during microbial cell invasion. *Microbes Infect* **2,** 85-89.

Weiss, G. (2002). Iron and immunity: a double-edged sword. *Eur J Clin Invest* **32 Suppl 1,** 70-78.

Weiss, G. (2005). Modification of iron regulation by the inflammatory response. *Best Pract Res Clin Haematol* **18,** 183-201.

Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G.*, et al.* (1993). Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *Embo J* **12,** 3651-3657.

Weiss, G., Meusburger, E., Radacher, G., Garimorth, K., Neyer, U. and Mayer, G. (2003). Effect of iron treatment on circulating cytokine levels in ESRD patients receiving recombinant human erythropoietin. *Kidney Int* **64,** 572-578.

Weiss, G., Umlauft, F., Urbanek, M., Herold, M., Lovevsky, M., Offner, F. and Gordeuk, V.R. (1999). Associations between cellular immune effector function, iron metabolism, and disease activity in patients with chronic hepatitis C virus infection. *J Infect Dis* **180,** 1452-1458.

Weiss, G., Werner-Felmayer, G., Werner, E.R., Grunewald, K., Wachter, H. and Hentze, M.W. (1994). Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med* **180,** 969-976.

Winkelmann, G. (2002). Microbial siderophore-mediated transport. *Biochem Soc Trans* **30,** 691-696.

Zaharik, M.L., Cullen, V.L., Fung, A.M., Libby, S.J., Kujat Choy, S.L., Coburn, B.*, et al.* (2004). The Salmonella enterica serovar typhimurium divalent cation transport systems MntH and SitABCD are essential for virulence in an Nramp1G169 murine typhoid model. *Infect Immun* **72,** 5522-5525.

Zaharik, M.L., Vallance, B.A., Puente, J.L., Gros, P. and Finlay, B.B. (2002). Host-pathogen interactions: Host resistance factor Nramp1 up-regulates the expression of Salmonella pathogenicity island-2 virulence genes. *Proc Natl Acad Sci U S A* **99,** 15705-15710.

Zwilling, B.S., Kuhn, D.E., Wikoff, L., Brown, D. and Lafuse, W. (1999). Role of iron in Nramp1-mediated inhibition of mycobacterial growth. *Infect Immun* **67,** 1386-1392.

Figure legends

Figure 1.

Effects of Slc11a1 on iron acquisition and iron release by Salmonella-infected macrophages

Slc11a1-non-functional RAW-21 cells (light grey bars) and Slc11a1-functional RAW-37 cells (dark grey bars) were infected with *S.* Typhimurium (S. tm.) at a MOI of 10 and stimulated with 50 U/ml IFN-γ for 24 hours. TfR1 and Fpn1 mRNA levels (Figures 1A and 1C, respectively) were determined by qPCR. Values were corrected for the amount of 18S ribosomal RNA, which was determined in parallel. Results are shown as relative differences of this ratio in comparison to unstimulated Slc11a1-expressing RAW-37 control macrophages (=1.0). Data are expressed as mean ± SD of five independent experiments. The uptake of TBI as well as iron release (depicted in Figures 1B and 1D and, respectively) were determined as described in 'Experimental procedures'. Data are shown as mean ± SD of five independent experiments performed in duplicates and are expressed as fold change in the relative iron uptake/release in comparison to RAW-37 controls (=1.0). * $P < 0.05$ for infected/stimulated RAW-21 macrophages in comparison to RAW-21 controls; ° $P < 0.05$ for infected/stimulated RAW-37 in comparison to untreated RAW-37 cells; # $P < 0.05$ for the comparison of RAW-37 and RAW-21 cells subjected to the same treatment.

Figure 2.

Regulation of TfR1 and Fpn1 protein levels in Salmonella-infected RAW-21 and RAW-37 cells

Slc11a1-non-expressing RAW-21 cells and Slc11a1-expressing RAW-37 cells were infected with *Salmonella* and stimulated with IFN-γ exactly as described in the legend to Figure 1. Whole cell lysates were analysed by immunoblotting using specific antibodies to TfR1 (upper panel) and Fpn1 (middle panel). Equal loading of protein extracts was confirmed by re-

probing membranes with an anti-Actin antibody (lower panel). One of four representative immunoblot experiments is shown.

Figure 3.

Effects of Slc11a1 on ferritin expression and on intracellular iron content in Salmonella-infected macrophages

RAW-21 and RAW-37 cells were infected with *Salmonella* and activated with IFN-γ exactly as described in the legend to Figure 1. The mRNA levels of H-ferritin were determined by qPCR (Figure 3A). Results are shown as relative differences of this ratio in comparison to the unstimulated control macrophages (=1.0). The total cellular iron content was determined by means of atomic absorption spectrometry and normalized for the protein content (Figure 3B). Data are expressed as means ± SD of five independent experiments. Statistically significant differences are indicated as described in the legend to figure 1.

Figure 4.

Impact of Slc11a1 on macrophage immune effector functions after IFN-γ stimulation

RAW-21 and RAW-37 cells were stimulated with IFN-γ and/or infected with *Salmonella* for 24 hours. Culture supernatants were analyzed for RNS (as determined by measuring nitrite levels, Figure 4A), TNF-α (Figure 4B), IL-6 (Figure 4C) and IL-10 (Figure 4D) as described in 'Experimental procedures'. Data are shown as mean ± SD of four to five independent experiments. Statistically significant differences are indicated as described in the legend to figure 1.

Figure 5.

Impact of Slc11a1 on the intracellular survival of Salmonella

RAW-21 and RAW-37 cells were infected with *Salmonella* for the indicated periods (Figure 5A) or infected for 24 hours and activated with IFN-γ (Figure 5B). Bacterial load was determined by selective plating as described in 'Experimental procedures'.

Figure 6.

Impact of Slc11a1 on macrophage immune effector functions in the presence of exogenous iron perturbations

In additional experiments, macrophages were infected with *Salmonella* and exposed to serial concentrations of human holo-transferrin. Bacterial load was determined by selective plating (Figure 6A). Cell culture supernatants were analyzed for the formation of nitrite, TNF-α, IL-10 and IL-6 as described (Figures 6B, C, D and E, respectively). Statistically significant differences are indicated as described

Figure 7.

Contributions of Fpn1-mediated iron export and of TBI-uptake to Slc11a1-dependent macrophage functions

RAW-21 and RAW-37 cells were pre-treated for 8 hours with $FeSO_4$, DFO, synthetic Hamp, a blocking α-TfR1 antibody or the appropriate control. Subsequently, cells were infected with *Salmonella* for 12 hours. Intracellular bacteria were enumerated (Figure 7A) and concentrations of TNF-α (Figure 7B) and of nitrite (Figure 7C) in supernatants were measured by a specific ELISA or the Griess reaction, respectively.

Figure 8.

Limitation of iron availability for intramacrophage Salmonella in the presence of Slc11a1

The acquisition of NTBI (Figure 8A) and TBI (Figure 8B) by intramacrophage bacilli was determined as described in 'Experimental procedures' using *S.* Typhimurium engulfed by

Slc11a1-non-functional RAW-21 in comparison to Slc11a1-functional RAW-37 phagocytes. Data are shown as means ± SD of five independent experiments and normalized for the number of bacteria as determined by selective plating.

Figure 9.

Antibacterial effects in RAW-21 and RAW-37 cells infected with iron-uptake mutant Salmonella

RAW-21 and RAW-37 phagocytes were infected with *S.* Typhimurium wild-type strain as well as with isogenic *entC* and *entC sit feo* mutant derivatives and treated with PBS or 12.5 μg/ml holo-transferrin. The intracellular survival of bacteria was enumerated after 20 hours, and data are presented as relative values in comparison to solvent-treated RAW-37 cells infected with wild-type *Salmonella* (Figure 9A). Culture supernatants were analyzed for the concentrations of TNF-α (Figure 9B) and nitrite (9C).

Acknowledgements

We thank Sabine Engl and Markus Seifert for excellent technical support.

Financial support by the 'Verein zur Förderung von Forschung und Weiterbildung in molekularer Immunologie und Infektiologie an der Medizinischen Universität Innsbruck', the Austrian research Funds FWF, Project 'P-19664' (to G. W.), the European Union, Project 'Euroiron 1' (to G. W.) and the National Institutes of Health (to F. C. F.) is gratefully acknowledged.

Figures

Figure 1.

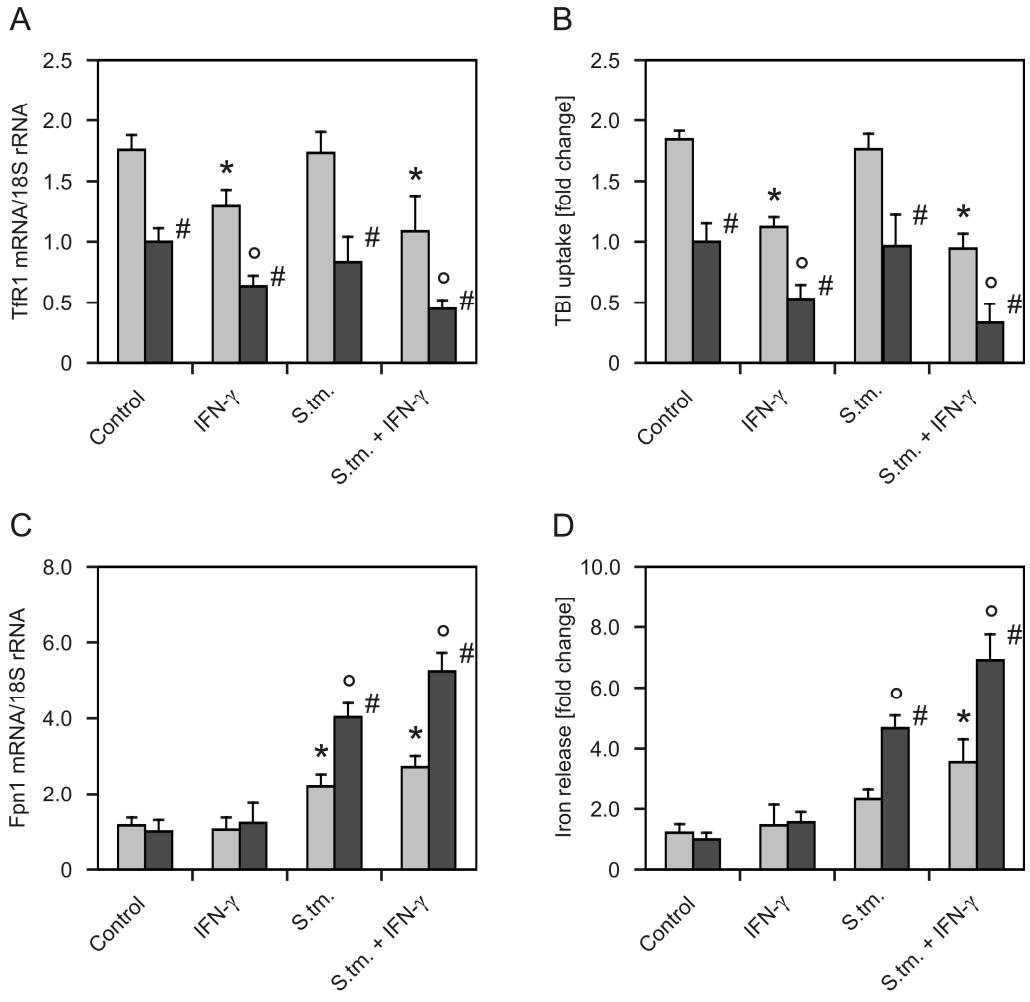


Figure 2.

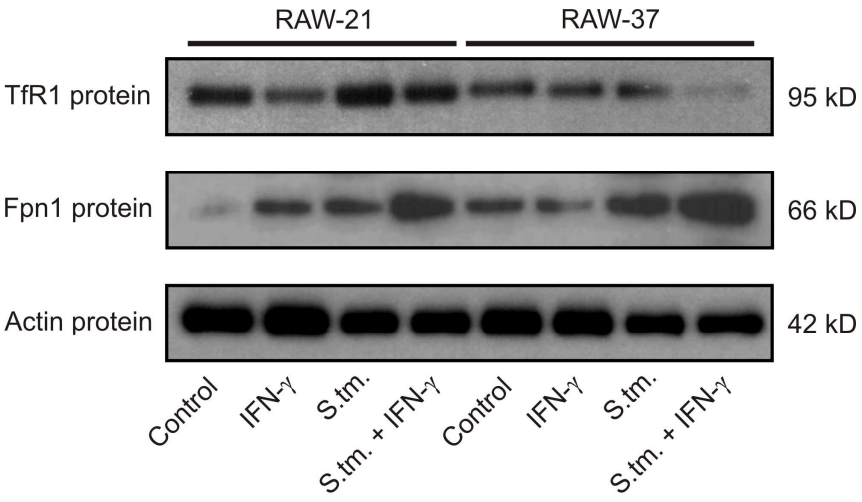

RAW-21
RAW-37
TfR1 protein
95 kD
Fpn1 protein
66 kD
Actin protein
42 kD
Control
IFN-γ
S.tm.
S.tm. + IFN-γ
Control
IFN-γ
S.tm.
S.tm. + IFN-γ

Figure 3.

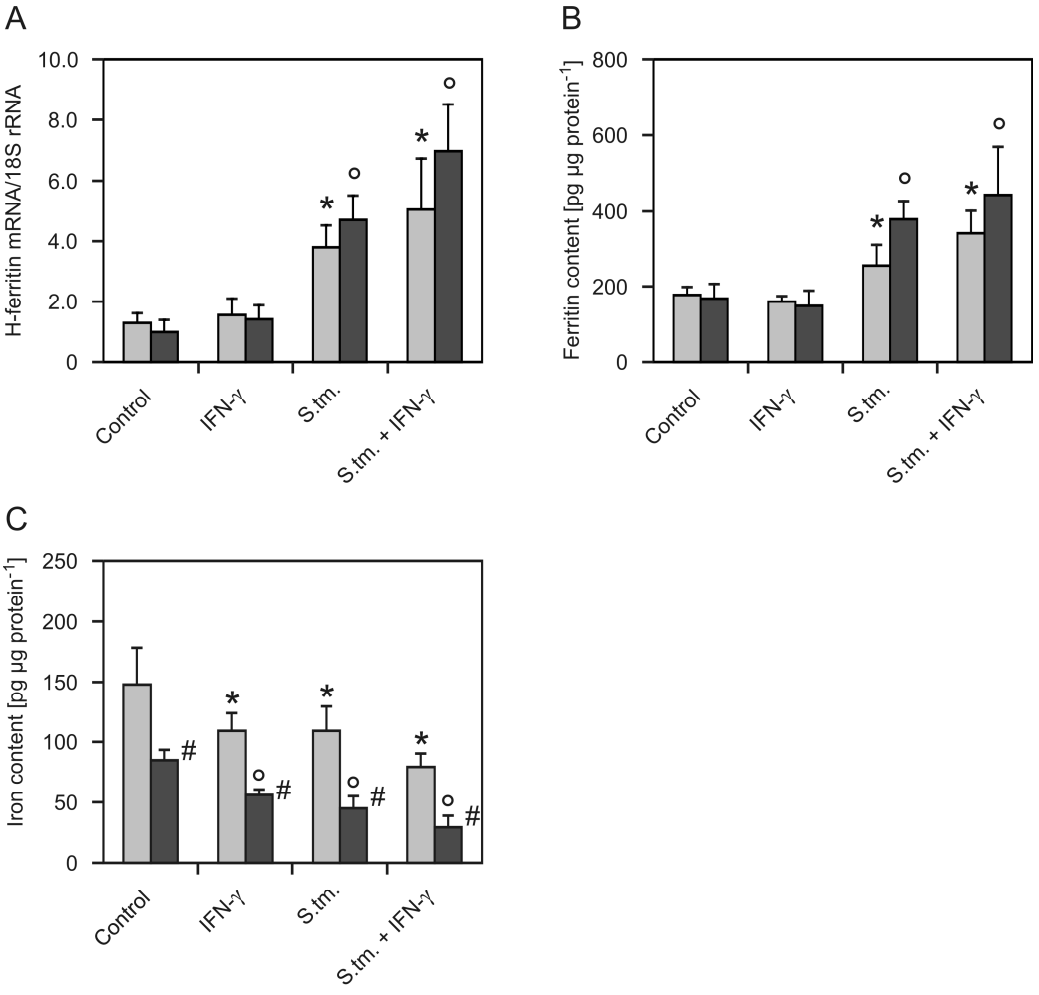

A
H-ferritin mRNA/18S rRNA
10.0
8.0
6.0
4.0
2.0
0
Control
IFN-γ
S.tm.
S.tm. + IFN-γ
B
Ferritin content [pg µg protein-1]
800
600
400
200
0
Control
IFN-γ
S.tm.
S.tm. + IFN-γ
C
Iron content [pg µg protein-1]
250
200
150
100
50
0
Control
IFN-γ
S.tm.
S.tm. + IFN-γ

Figure 4.

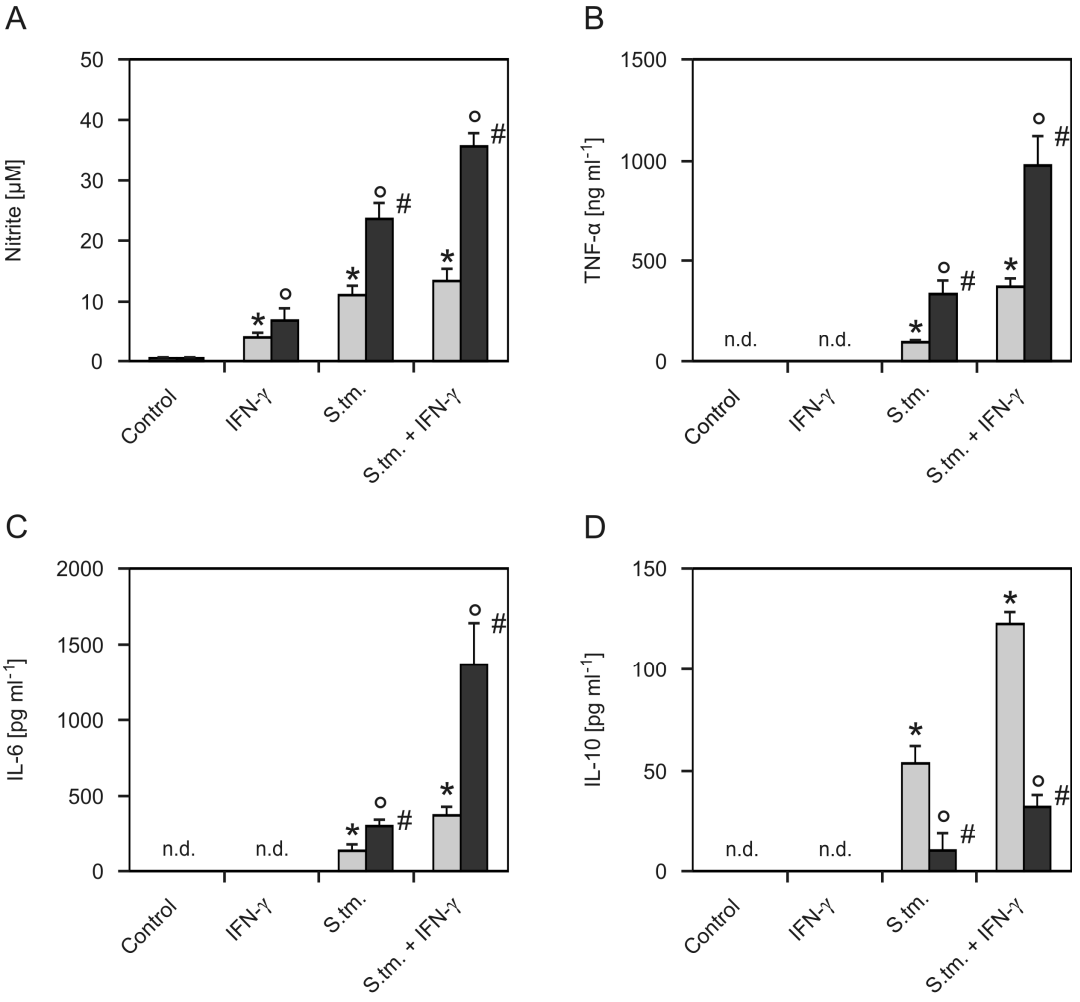

A
Nitrite [µM]
50
40
30
20
10
0
Control
IFN-γ
S.tm.
S.tm. + IFN-γ
B
TNF-α [ng ml-1]
1500
1000
500
0
n.d.
n.d.
C
IL-6 [pg ml-1]
2000
1500
1000
500
0
D
IL-10 [pg ml-1]
150
100
50
0

Figure 5.

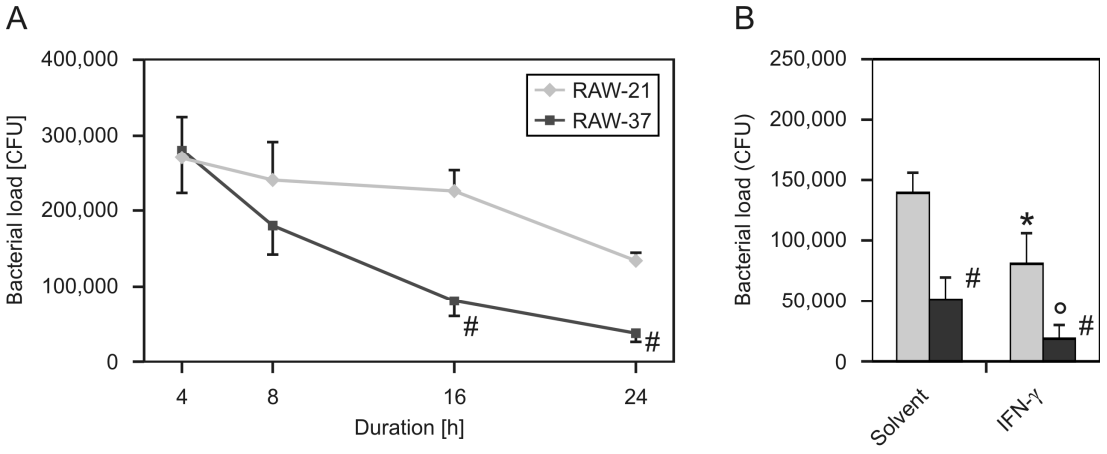

A
400,000
300,000
200,000
100,000
0
Bacterial load [CFU]
RAW-21
RAW-37
#
#
4
8
16
24
Duration [h]
B
250,000
200,000
150,000
100,000
50,000
0
Bacterial load (CFU)
#
*
°
#
Solvent
IFN-γ

Figure 6.

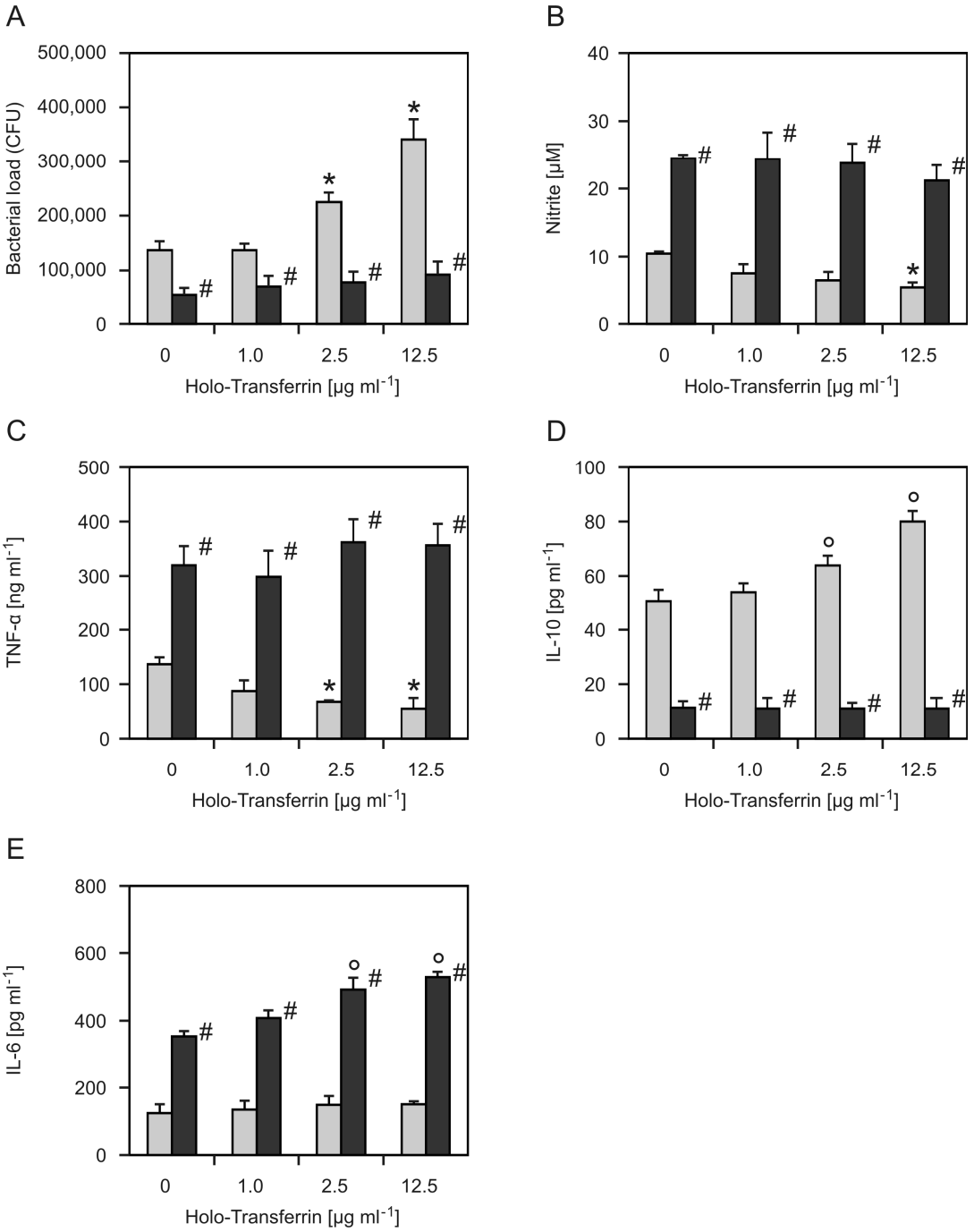

A
Bacterial load (CFU)
500,000
400,000
300,000
200,000
100,000
0
0
1.0
2.5
12.5
Holo-Transferrin [µg ml-1]
B
Nitrite [µM]
40
30
20
10
0
Holo-Transferrin [µg ml-1]
C
TNF-α [ng ml-1]
500
400
300
200
100
0
Holo-Transferrin [µg ml-1]
D
IL-10 [pg ml-1]
100
80
60
40
20
0
Holo-Transferrin [µg ml-1]
E
IL-6 [pg ml-1]
800
600
400
200
0
Holo-Transferrin [µg ml-1]

Figure 7.

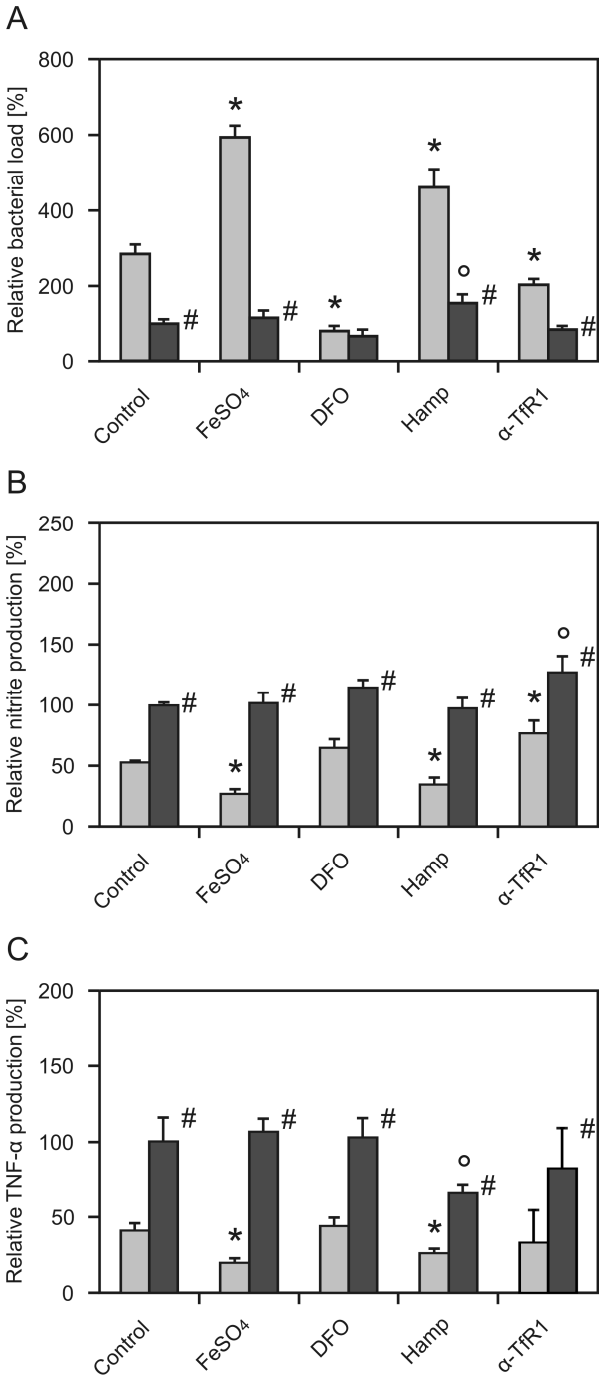

A
Relative bacterial load [%]
800
600
400
200
0
Control
FeSO4
DFO
Hamp
α-TfR1
B
Relative nitrite production [%]
250
200
150
100
50
0
C
Relative TNF-α production [%]
200
150
100
50
0

Figure 8.

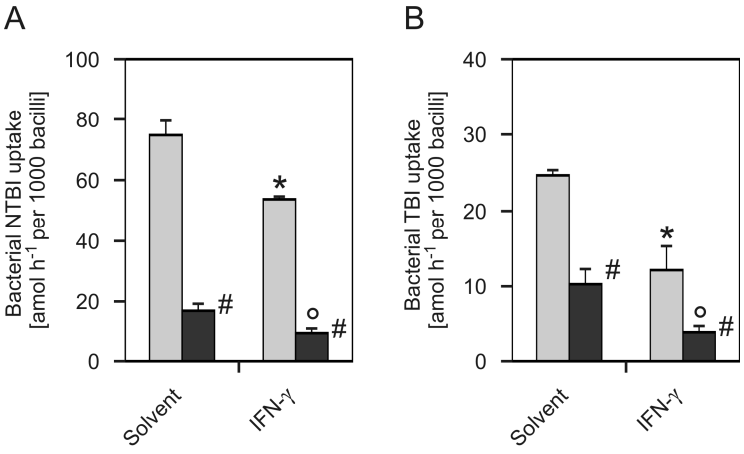
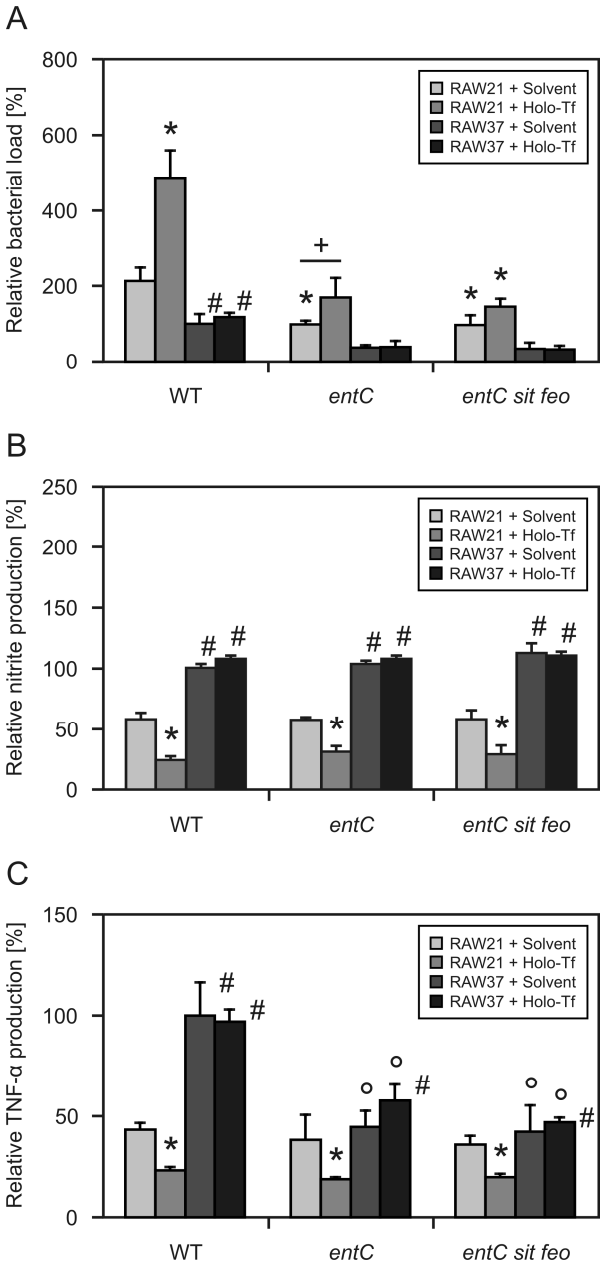

A
B
Bacterial NTBI uptake
[amol h-1 per 1000 bacilli]
Bacterial TBI uptake
[amol h-1 per 1000 bacilli]
100
80
60
40
20
0
40
30
20
10
0
*
#
°
#
Solvent
IFN-γ

Figure 9.


A
Relative bacterial load [%]
800
600
400
200
0
RAW21 + Solvent
RAW21 + Holo-Tf
RAW37 + Solvent
RAW37 + Holo-Tf
WT
entC
entC sit feo
B
Relative nitrite production [%]
250
200
150
100
50
0
C
Relative TNF-α production [%]
150
100
50
0