JLB

Brief Conclusive Report

Slc11a1 (Nramp1) impairs growth of *Salmonella enterica* serovar *typhimurium* in macrophages via stimulation of lipocalin-2 expression

Gernot Fritsche, Manfred Nairz,* Stephen J. Libby,† Ferric C. Fang,† and Günter Weiss*,1*

*Department of Internal Medicine I, Clinical Immunology and Infectious Diseases, Innsbruck Medical University, Innsbruck, Austria; and †Departments of Laboratory Medicine and Microbiology, University of Washington School of Medicine, Seattle, Washington, USA

RECEIVED NOVEMBER 12, 2011; REVISED MAY 22, 2012; ACCEPTED MAY 24, 2012. DOI: 10.1189/jlb.1111554

ABSTRACT

The expression of the cation transporter Nramp1 (Slc11a1) in late phagolysosomes confers resistance to infection with several intracellular pathogens, such as *Salmonella enterica*, in mice. The antimicrobial actions of Nramp1 are attributable, in part, to modulation of macrophage immune function and cellular iron metabolism—the latter affecting the availability of the essential nutrient iron for intraphagosomal bacteria. Here, we provide novel evidence that Nramp1 functionality increases the expression of the peptide Lcn2, which exerts its antimicrobial activity by scavenging iron-loaded bacterial siderophores and mediating iron efflux from macrophages. With the use of macrophage cell lines expressing functional or nonfunctional Nramp1, we found significantly elevated Lcn2 mRNA and protein levels in Nramp1-expressing cells. These resulted from Nramp1-mediated alterations in the production of ROS, which stimulated NF-κB activity and subsequently, Lcn2 transcription. We observed that increased Lcn2 levels in primary Nramp1-positive macrophages resulted in a significant suppression of *S. enterica* serovar *typhimurium* growth. Stimulation of Lcn2 expression is a novel mechanism by which Nramp1 confers resistance against infection with the intracellular bacterium *S. typhimurium*. *J. Leukoc. Biol.* 92: 353–359; 2012.

Introduction

Innate resistance to infection with a number of intracellular microbes is strongly influenced by the expression of Nramp1—also called Slc11a1. Nramp1 is a protein expressed in the membranes of late phagolysosomes of macrophages, as well as neutrophils and DCs [1–3]. In mice, the function of Nramp1 is abrogated by a single amino acid substitution, leading to a fatal course of infection with intracellular pathogens, such as *Salmonella*, *Mycobacterium*, and *Leishmania spp.* [4]. In humans, *NRAMP1* polymorphisms are associated with increased susceptibility to tuberculosis [5] and certain autoimmune disorders [6].

Nramp1 exerts pleiotropic effects on macrophage function, which can be partly referred to its cation transport capacity [7, 8]. Nramp1 functionality alters intramacrophage iron homeostasis [9–11], as evidenced by reduced cytoplasmic iron levels, enhanced cellular iron export, and most importantly, limited availability of iron for intracellular bacteria, as demonstrated for *Salmonella* or *Mycobacterium* residing within the phagolysosome [11–13]. Nramp1 has also been shown to be involved in macrophage iron recycling following erythrophagocytosis [14].

Nramp1 functionality is associated with an enhanced activity of various proinflammatory immune pathways, as evidenced by the increased production of antimicrobial effector molecules, such as ROS and RNS [15–17]. On the other hand, Nramp1 down-regulates the expression of the anti-inflammatory cytokine IL-10, which also leads to enhanced NO production [18].

Lcn2, also known as siderocalin, 24p3, or neutrophil gelatinase-associated Lcn, is a small peptide produced by neutrophils, macrophages, and epithelial cells [19]. Lcn2 has been shown to exert bacteriostatic effects that have been attributed to its ability to bind iron-loaded bacterial siderophores [20], such as enterobactin and carboxymycobactins [21, 22]. Siderophores are part of an important iron-uptake mechanism for bacteria to ensure a sufficient supply of this nutrient. Lcn2 excretion by the host interferes with siderophore-mediated bacterial iron acquisition, which is an important defense mechanism against infections with enteric bacteria, including *Escherichia coli* and *Salmonella* [21, 23, 24]. In addition, Lcn2 may affect host iron homeostasis via binding of mammalian sidero-

Abbreviations: CAPE=caffeic acid phenethyl ester, DFO=desferrioxamine, HIF-1=hypoxia-inducible factor-1, HPRT=hypoxanthine phosphoribosyltransferase, L-NIL=L-N6-(1-iminoethyl)-lysine, Lcn2=lipocalin-2, Nramp1=natural resistance-associated macrophage protein 1, RNS=reactive nitrogen species, Slc11a1=solute carrier family 11 member 1, YC-1=3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole

1. Correspondence: Dept. of Internal Medicine I, Clinical Immunology and Infectious Diseases, Medical University of Innsbruck, Anichstrasse 35, A-6020, Innsbruck, Austria. E-mail: guenter.weiss@i-med.ac.at

phores [25, 26]. Induction of Lcn2 expression promotes iron release from macrophages, thereby impeding iron acquisition and growth of intramacrophage *Salmonella* and increasing host survival in bacterial sepsis [27]. Accordingly, Lcn2-mediated protection against bacterial infection can be counteracted by the addition of excess iron or siderophores, such as enterobactin [28].

As *Salmonella* infection is controlled not only by Nramp1-functionality but also by Lcn2, we questioned whether part of the protective effect of Nramp1 against this bacterial infection might be related to changes in Lcn2 expression. For this purpose, we used murine RAW264.7 macrophages that have been stably transfected with functional Nramp1 or nonfunctional antisense constructs and primary macrophages from Nramp1-susceptible and -resistant mice to look for possible differences in Lcn2 expression and to elucidate the underlying mechanisms.

MATERIALS AND METHODS

Cell culture

RAW-264.7 murine macrophages originating from Nramp1-susceptible BALB/c mice were stably transfected with an expression vector containing Nramp1 in the sense direction (RAW-37) or with a nonfunctional antisense construct (RAW-21), as described previously [29]. Nramp1 protein expression in RAW-37 macrophages was verified by Western blotting. Primary peritoneal macrophages differing in Nramp1 and/or Lcn2 expression were isolated from various mouse strains on a C57BL/6 background, as described previously [21, 30]. Nramp1+ mice (from Philippe Gros) and Lcn2-deficient mice (from Kelly Smith) on a C57BL/6 background were crossed to derive Nramp1+ Lcn2-deficient progeny that were confirmed by PCR genotyping.

Mice were housed under specific pathogen-free conditions at the animal facility of the University of Washington School of Medicine (Seattle, WA, USA). Isolation of primary peritoneal macrophages was approved by the institutional animal care and use committee and performed according to institutional guidelines. Age- and sex-matched mice were i.p.-injected with 0.5 ml 4% Brewer's thioglycollate medium (Sigma-Aldrich, Vienna, Austria) for 3 days. Mice were sacrificed by carbon dioxide inhalation and cells harvested by flushing the peritoneal cavity with PBS. Cells were seeded in six-well dishes in complete DMEM. Subsequently, nonadherent cells were removed by extensive washing with PBS, and adherent peritoneal macrophages were treated and analyzed as specified.

For stimulation of cells, the following reagents were used: murine IFN-γ (R&D Systems, Abingdon, UK); alloxan, catalase, LPS, and SOD (Sigma-Aldrich); and L-NIL (Alexis Biochemicals/Eubio, Vienna, Austria). For some experiments, cells were treated with YC-1 or CAPE (Marligen Biosciences, Rockville, MD, USA). Both substances were diluted in DMSO (Sigma-Aldrich), which was also used as a solvent control.

ELISA

Concentrations of murine Lcn2 in cell culture supernatants were measured by a commercially available ELISA kit (R&D Systems), strictly according to the manufacturer's instructions.

In vitro infection experiments

Unless otherwise specified, infection experiments were carried out using *S. typhimurium* WT, strain ATCC 14028s. In some experiments, an isogenic derivative containing an *entC*::*aph* mutation, which eliminates the ability to produce catecholate-type siderophores, was used. Strains were constructed, grown as described [31], and used as follows.

Bacteria were grown in LB broth to late logarithmic phase before cells (1×10^6 cells seeded into six-well plates in 2 ml DMEM culture medium) were infected with *Salmonella* bacteria at a multiplicity of infection of 10, as described [32]. After incubation for 1 h, cells were washed three times with PBS and replenished with DMEM, supplemented with 16 μg/ml gentamicin (Sandoz, Kundl, Austria) to kill extracellular bacteria. Macrophages were then stimulated with an anti-mouse neutralizing Lcn2 mAb (R&D Systems) or the iron chelator DFO (Sigma-Aldrich) for 24 h. For quantification of intracellular bacteria, cells were washed five times with PBS, lysed with 0.5% deoxycholic acid (Sigma-Aldrich), and plated onto LB agar plates for counting.

Quantitative real-time RT-PCR

TaqMan quantitative real-time RT-PCR analysis was done exactly as described [33]. Amplification conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The following probes (5′FAM- and 3′black hole quencher-1-labeled) were used for murine Lcn2: primer forward, 5′-GCC TCA AGG ACG ACA ACA TCA-3′, primer reverse, 5′-CAC CAC CCA TTC AGT TGT CAA T-3′; TaqMan: 5′-TTC TCT GTC CCC ACC GAC CAA TGC-3′. Data were normalized to HPRT expression.

NF-κB assay

Nuclear extracts were prepared as described previously [34]. NF-κB p65 binding activity was measured by a commercial chemiluminescence transcription factor kit (Thermo Scientific, Vienna, Austria).

Statistics

Statistical analyses for significant differences between groups were done by two-tailed Student's *t* test using SPSS software (Version 18; Chicago, IL, USA).

RESULTS AND DISCUSSION

Lcn2 expression is increased in cells expressing Nramp1

To examine whether Nramp1 functionality alters Lcn2 production, we first measured Lcn2 mRNA and protein expression in Nramp1-functional (RAW-37) or -nonfunctional (RAW-21) murine macrophages. After stimulation with IFN-γ and LPS for 24 h, Lcn2-mRNA levels were elevated significantly in Nramp1-expressing cells, as determined by quantitative real-time PCR (**Fig. 1A**), which translated into comparable differences in Lcn2-protein levels measured by ELISA in cell culture supernatants (Fig. 1B). Dose-response experiments with increasing doses of IFN-γ and LPS revealed that LPS is the main stimulus for Lcn2 production. However, whereas IFN-γ alone had no stimulatory potential for Lcn2 expression, it synergized with LPS to enhance Lcn2 mRNA expression (**Fig. 2A**). To verify this result in a different model, we examined Lcn2 expression in peritoneal macrophages from Nramp1-susceptible C57BL/6 mice or congenic Nramp1-resistant mice. Peritoneal macrophages were stimulated ex vivo with IFN-γ plus LPS, and Lcn2 levels in supernatants were measured by ELISA. Again, Nramp1-expressing macrophages produced significantly more Lcn2 than cells lacking functional Nramp1 (Fig. 2B).

Lcn2 restricts intramacrophage growth of *Salmonella* by limiting iron availability

To verify whether the observed differences in Lcn2 expression are functionally relevant, we investigated the growth of *S. typhi-*

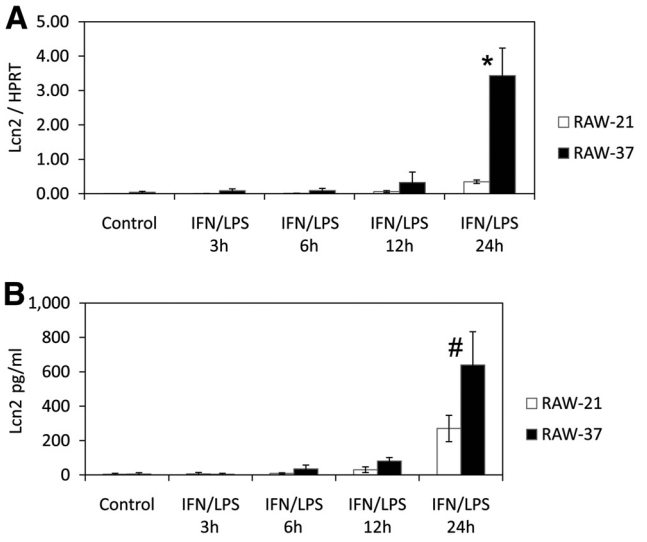

Figure 1. Lcn2-mRNA (A) and Lcn2-protein (B) levels in RAW-21 (Nramp1-negative) and RAW-37 (Nramp1-expressing) macrophages. Cells were left untreated or stimulated with IFN-γ (50 U/ml) and LPS (10 ng/ml) for 3–24 h, and Lcn2 mRNA and protein expression were quantified by RT-PCR or ELISA. Data are shown as means ± SD of four independent experiments. $^{*}P < 0.01$; $^{\#}P < 0.05$ when comparing RAW-21 with RAW-37 under the same treatment conditions.

murium in Nramp1-functional and -nonfunctional RAW macrophages. Initial uptake of bacteria did not differ between the cell lines (data not shown). As expected and described previously [18], the intramacrophage growth of *Salmonella* was suppressed in Nramp1-expressing RAW-37 cells as compared with RAW-21 cells. Interestingly, neutralization of Lcn2 by addition of an inhibitory anti-Lcn2 antibody strongly impaired the ability of both cell lines to control *Salmonella* growth, yielding similar bacterial counts in RAW-21 and RAW-37 cells (**Fig. 3A**). This effect of the anti-Lcn2 antibodies can be attributed to increased iron availability for bacterial growth, as addition of the iron chelator DFO abolished *Salmonella* growth almost completely in both cell lines, even when Lcn2 was neutralized. Accordingly, when we studied the infection of macrophages using an *entC* mutant *Salmonella* strain unable to produce the bacterial siderophore enterobactin, which is the target structure for Lcn2, we found that the growth of bacteria within macrophages was strongly reduced. Importantly, the growth of Δ*entC Salmonella* could not be restored by the addition of a Lcn2-neutralizing antibody (Fig. 3A). Independent from the used bacterial strain, growth was suppressed to a minimum upon iron chelation with DFO.

We verified the importance of Lcn2 for control of *Salmonella* growth in ex vivo experiments with peritoneal macrophages taken from C57BL/6 mouse strains differing in Nramp1 and/or Lcn2 expression. Again, Nramp1+ mice significantly suppressed growth of WT *S. typhimurium* compared with Nramp1-susceptible C57BL/6 mice, even in macrophages obtained from Lcn2 knockout mice. However, maximum growth inhibition was observed in Nramp1+/Lcn2+ macrophages, which was significantly better than in macrophages taken from Nramp1-expressing, Lcn2-knockout mice. Again, these findings could be traced back to limited availability of iron, as all of these effects were abolished when an *entC* mutant *Salmonella* strain was used (Fig. 3B).

Increased Lcn2 levels in Nramp1-expressing cells are related to hydrogen peroxide production

As elevated Lcn2 production by Nramp1-expressing cells appeared to be an important factor for controlling intramacrophage bacterial growth, we sought to identify the mechanisms by which Nramp1 influences Lcn2 expression. As Nramp1 also acts as an iron transporter, we examined whether differences in Lcn2 production can be attributed to differences in cellular iron levels between RAW-21 and -37 cells [11]. However, the significant differences in Lcn2 mRNA expression between RAW-21 and -37 cells persisted, despite iron supplementation or chelation using DFO (data not shown). This was also true when intracellular iron levels were increased by the addition of recombinant hepcidin, which impairs cellular iron export (data not shown). As the two cell lines strongly differ in the production of reactive oxygen and nitrogen inter-

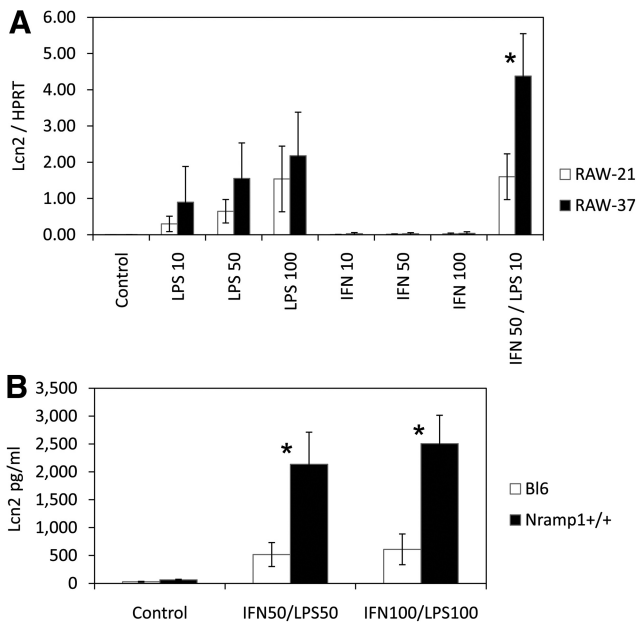

Figure 2. Lcn2 expression in transfectants and in murine peritoneal macrophages. (A) IFN-γ/LPS dose-response experiments. RAW-21/RAW-37 cells were left untreated or stimulated with increasing doses of LPS (ng/ml) and/or IFN-γ (U/ml) for 24 h, and Lcn2 mRNA expression was quantified by RT-PCR. Data are shown as means ± SD of three independent experiments performed in triplicates at the same time-point and expressed as relative Lcn2 mRNA abundance after normalization for HPRT. $^{*}P < 0.05$ when comparing RAW-21 with RAW-37 under the same treatment conditions. (B) Lcn2 protein levels in supernatants of murine peritoneal macrophages taken from C57BL/6 mice (Nramp1-susceptible) or congenic Nramp1-resistant (Nramp1+/+) mice. Cells were left untreated or stimulated with increasing doses of LPS (ng/ml) and/or IFN-γ (U/ml) for 24 h before Lcn2 protein expression was quantified by ELISA. Data are shown as means ± SD of five independent experiments. $^{*}P < 0.05$.

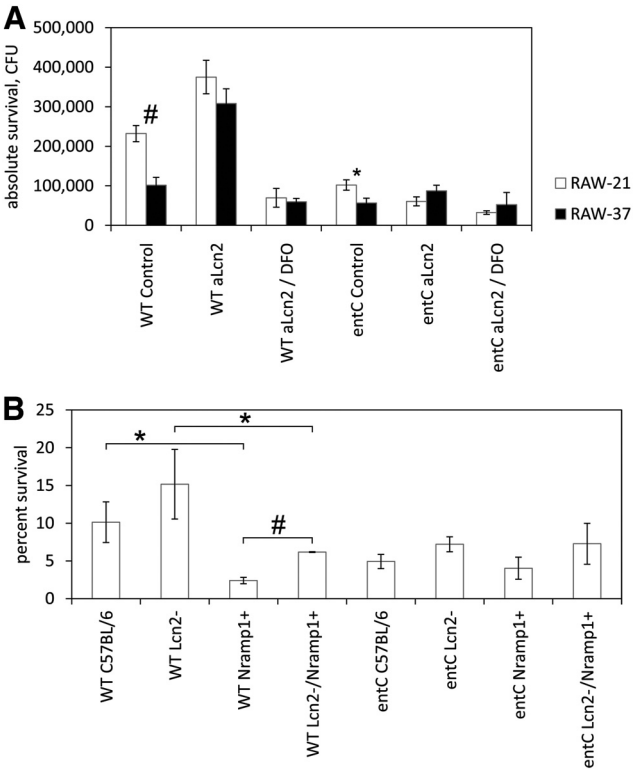

Figure 3. Growth of *S. typhimurium* in macrophages. (A) RAW-21/RAW-37 cells were infected with *S. typhimurium* and treated with a neutralizing anti-Lcn2 (aLcn2) antibody (25 μg/ml) or the iron chelator DFO (100 μM), and growth of *S. typhimurium* was quantified as detailed in Materials and Methods. Effects were examined on WT *Salmonellae* as well as *S. typhimurium* carrying a nonfunctional mutation of the bacterial siderophore enterobactin (entC). Data are expressed as means ± SD of three independent experiments. $^{\#}P < 0.01$; $^{*}P < 0.05$ when comparing RAW-21 (Nramp1-negative) with RAW-37 (Nramp1-positive). (B) Growth of WT and enterobactin-deficient *Salmonella* in peritoneal macrophages taken from C57BL/6 mice differing in Nramp1 and/or Lcn2 expression, as described in Materials and Methods. *Salmonella* survival is shown relative to initial bacterial counts after infection. Data are shown as means ± SD of three independent experiments. $^{\#}P < 0.01$; $^{*}P < 0.05$.

mediates as a function of their Nramp1 phenotype [15], we next investigated whether this accounted for differential Lcn2 expression. Interestingly, the addition of catalase, acting as a scavenger of hydrogen peroxide, led to significantly reduced Lcn2-mRNA expression in RAW-37 cells, which was then comparable with the Lcn2 mRNA levels observed in RAW-21 cells (**Fig. 4**). In contrast, neither the addition of SOD (a scavenger of superoxide) nor L-NIL (an inhibitor of iNOS) significantly influenced Lcn2 mRNA expression in RAW-21 and -37 cells.

NF-κB activation is responsible for increased Lcn2 production in Nramp1-expressing cells

We concluded that a hydrogen peroxide-sensitive transcription factor may account for differences in Lcn2 expression. Therefore, we examined the effect of specific pharmacological inhibitors, i.e., YC-1 and CAPE, which block the activity of the transcription factors HIF-1 and NF-κB, respectively. Whereas inhibiton of HIF-1α activity did not affect the differences in Lcn2 expression between RAW-21 and -37 cells, the application of CAPE reduced Lcn2 expression to comparable levels in both cell lines, indicating that Nramp1 mediates Lcn2 expression via increased transcriptional activity of NF-κB (**Fig. 5**). To confirm that NF-κB is responsible for Lcn2 up-regulation in Nramp1-functional cells, we measured NF-κB activity using a transcription factor activity assay. Within the first hour after cytokine stimulation, NF-κB activity was significantly higher in RAW-37 macrophages. Moreover, when cells were treated additionally with alloxan, a potent inducer of ROS, NF-κB activity in RAW-21 was strongly increased to levels similar to those observed in RAW-37 cells (**Fig. 6A**).

Accordingly, following alloxan treatment, the Lcn2 mRNA expression in cytokine-stimulated RAW-21 cells was elevated to comparable levels, as seen in RAW-37 cells. As alloxan is a short-lived compound and thus, has to be added every 2 h, we only investigated cells that were stimulated for 6 h, a time-point at which

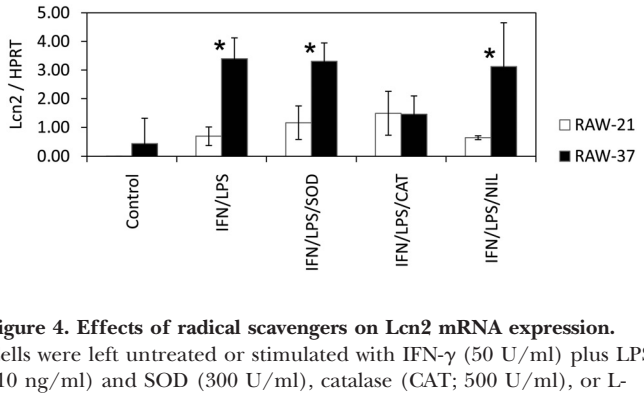

Figure 4. Effects of radical scavengers on Lcn2 mRNA expression. Cells were left untreated or stimulated with IFN-γ (50 U/ml) plus LPS (10 ng/ml) and SOD (300 U/ml), catalase (CAT; 500 U/ml), or L-NIL (600 μM) for 24 h. Data are mean ± SD of five independent experiments. $^{*}P < 0.05$ when comparing RAW-21 with RAW-37.

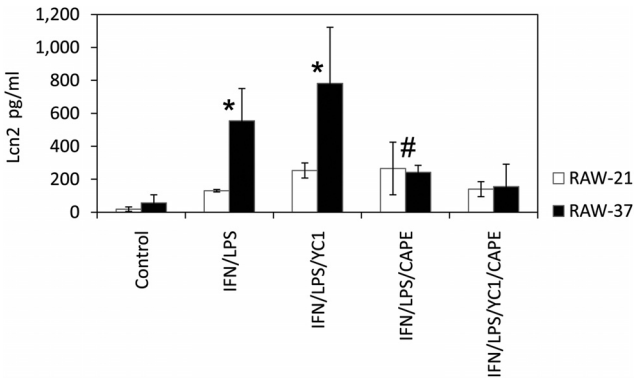

Figure 5. Effects of inhibitors of NF-κB and HIF-1 on Lcn2 production. Lcn2 levels in cell culture supernatants were determined by ELISA. Cells were left untreated or stimulated with IFN-γ (50 U/ml) plus LPS (10 ng/ml) and inhibitors of HIF-1 (YC-1; 5 μM) or NF-κB (CAPE; 12.5 μg/ml) for 24 h. Data are mean ± SD of three independent experiments. $^{*}P < 0.05$ when comparing RAW-21 with RAW-37; $^{\#}P < 0.05$ when comparing cytokine-stimulated RAW-37 cells with or without addition of CAPE.

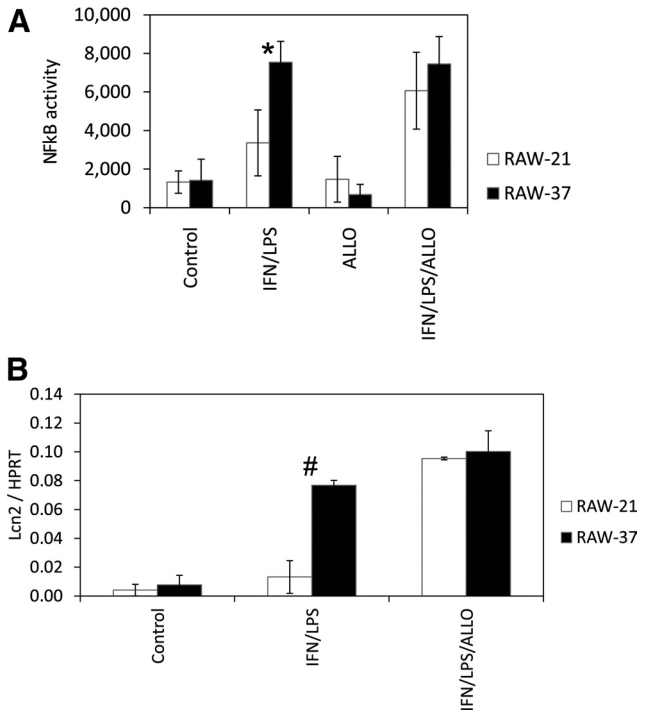

Figure 6. Effect of alloxan on NF-κB activity and Lcn2 expression. (A) Binding activity of NF-κB in nuclear extracts of macrophages. Cells were stimulated for 1 h with IFN-γ (50 U/ml) and LPS (10 ng/ml) and/or alloxan (ALLO; 0.1 mM). Nuclear extracts were prepared, and NF-κB binding assays were carried out, as detailed in Materials and Methods. Data are mean ± SD of seven independent experiments. $^{*}P < 0.05$ when comparing RAW-21 with RAW-37. (B) Lcn2-mRNA levels as quantified by RT-PCR. Cells were left untreated or stimulated with IFN-γ (50 U/ml) and LPS (10 ng/ml) for 6 h; alloxan (0.1 mM) was added repeatedly after 0, 2, and 4 h because of its short half-life. Data are shown as means ± SD of three independent experiments. $^{\#}P < 0.01$ when comparing RAW-21 with RAW-37 under the same treatment conditions.

differences in Lcn2 expression as a function of Nramp1 expression already became significantly different (Fig. 6B).

Nramp1 is considered an important component of the macrophage innate immune response, as it protects mice from infection with the intracellular pathogens *Salmonella*, *Mycobacterium*, and *Leishmania spp.* The antimicrobial effect of Nramp1 has been primarily attributed to two mechanisms. On one hand, Nramp1 acts as a transporter for divalent cations, thereby modulating phagolysosomal ion composition and the access of microbes to important divalent metals, such as manganese and iron [8, 35]. The limitation of iron availability inhibits bacterial growth and pathogenicity, whereas a reduction in manganese levels reduces the resistance of microbes to oxidative stress. On the other hand, Nramp1 functionality results in up-regulation of proinflammatory immune pathways and antimicrobial effector mechanisms [15], the latter reflected by increased production of ROS and RNS in Nramp1-expressing macrophages.

Herein, we provide evidence for a novel Nramp1-inducible resistance mechanism via increased expression of the peptide Lcn2, which exerts bacteriostatic effects via binding of iron-loaded bacterial siderophores, thus depriving bacteria from the essential growth factor iron. The antimicrobial effect of Lcn2 has been confirmed in a variety of bacterial pathogens that use siderophores for iron uptake, such as *E. coli* [21, 28], *Klebsiella* [36], and *Salmonella* [32]. Moreover, alveolar macrophages and epithelial cells of mice infected with mycobacteria up-regulate the expression of Lcn2, which inhibits mycobacterial growth in vitro and in vivo. The latter has been confirmed in $Lcn2^{-/-}$ mice infected with *Mycobacterium tuberculosis*, which exhibit impaired control of infection compared with $Lcn2^{+/+}$ littermates [37]. Lcn2-mediated inhibition of *M. tuberculosis* has been related to the binding of carboxymycobactins, which are soluble siderophores produced by mycobacteria [22]. Interestingly, in mycobacterial infection, the importance of Lcn2 is restricted to the early phase after inoculation, when Lcn2 impairs mycobacterial iron acquisition [38]. Recent data obtained in Nramp1-negative BALB/c mice infected with mycobacteria indicated that these mice developed anemia, which was associated with increased Lcn-2 expression as compared with Nramp1-expressing CD2 mice. Therefore, the induction of Lcn2 may result from increased pathogen numbers in BALB/c mice and may be considered as a compensatory mechanism to withdraw iron from pathogens and to overcome the impaired control of the infection as a result of lack of Nramp1 [39].

In the present study, we show that Nramp1 functionality leads to a significantly higher Lcn2 expression by macrophages. In agreement with previous observations, we found that LPS is the major stimulus for Lcn2 in macrophages [21], whereas the addition of IFN-γ is synergistic. Nramp1-induced differences in Lcn2 production were verified in primary peritoneal macrophages taken from congenic Nramp1-expressing or -nonexpressing mice.

Following infection with *S. typhimurium*, Nramp1-dependent differences in Lcn2 expression were found to be functionally important for host defense, and increased Lcn2 expression was accompanied by inhibition of *Salmonella* growth within macrophages. As the inhibitory effect of Nramp1 on intracellular *Salmonella* multiplication could be abolished by infection with an enterobactin-deficient *Salmonella* strain or the chelation of free iron, we conclude that the protective effect of Lcn2 can be attributed to inhibition of siderophore-mediated bacterial iron uptake. These findings are in agreement with data on Lcn2 function in bacterial infections [21, 40].

As Nramp1 works as a transporter of divalent ions, including iron, and Nramp1 functionality is associated with changes in iron homeostasis, which results in intramacrophage iron deficiency [9, 11], imbalances of iron homeostasis might be postulated to be responsible for Nramp1-dependent alterations in Lcn2 expression. However, we did not observe an association between intracellular iron availability and Lcn2 production (data not shown). In contrast, we provide evidence that Lcn2 formation is regulated by ROS. This is consistent with the earlier observation that Nramp1-expressing RAW cells produce significantly higher levels of ROS and RNS [15] relative to Nramp1-negative RAW cells. The effect of ROS on Lcn2 production can be related to radical-mediated activation of NF-κB

activity. In addition, NF-κB activity in Nramp1-negative cells can be stimulated upon the addition of alloxan, a potent inducer of ROS production, yielding NF-κB activation and Lcn2 expression to a similar extent as that observed in Nramp1-positive RAW cells. Our findings are consistent with previously published data showing that ROS, in particular, hydrogen peroxide, activate NF-κB [41].

Collectively, our observations show that a functional Nramp1 locus up-regulates the expression of the antimicrobial protein Lcn2 in murine macrophages. Lcn2 formation results from enhanced production of ROS by Nramp1-functional macrophages, leading to enhanced Lcn2 transcription via activation of the transcription factor NF-κB. Nramp1-dependent induction of Lcn2 leads to suppression of *S. typhimurium* growth in Nramp1-functional cells. Lcn2 acts by restricting enterobactin-mediated iron acquisition and thereby, depriving *Salmonella* of the essential nutrient iron. Modulation of Lcn2 is a novel mechanism by which Nramp1 promotes resistance to intracellular bacteria.

AUTHORSHIP

G.F. designed and performed experiments and analyzed data. M.N. performed infection experiments with *Salmonella* and analyzed data. S.J.L. and F.C.F. performed experiments using peritoneal mouse macrophages and analyzed data. G.W. designed the study and analyzed data. All authors contributed to the writing of the manuscript.

ACKNOWLEDGMENTS

This project was supported by Austrian Science Fund (FWF) grant TRP188 to G.W., as well as by the "Verein zur Förderung von Forschung und Weiterbildung in Infektiologie und Immunologie an der Medizinischen Universität Innsbruck". Support to F.C.F. and S.J.L. was provided by grants from the U.S. National Institutes of Health (AI39557, AI48622, AI77629). We thank Mrs. Sabine Engl for excellent technical assistance. RAW-21 and -37 cells were kindly provided by Dr. H. C. Barton (University of Southampton, UK). The authors are grateful to Kelly Smith (University of Washington) for providing Lcn2-deficient mice.

REFERENCES

1. Forbes, J. R., Gros, P. (2001) Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol.* **9,** 397–403.
2. Blackwell, J. M., Searle, S., Goswami, T., Miller, E. N. (2000) Understanding the multiple functions of Nramp1. *Microbes Infect.* **2,** 317–321.
3. Canonne-Hergaux, F., Calafat, J., Richer, E., Cellier, M., Grinstein, S., Borregaard, N., Gros, P. (2002) Expression and subcellular localization of NRAMP1 in human neutrophil granules. *Blood* **100,** 268–275.
4. Vidal, S., Tremblay, M. L., Govoni, G., Gauthier, S., Sebastiani, G., Malo, D., Skamene, E., Olivier, M., Jothy, S., Gros, P. (1995) The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *J. Exp. Med.* **182,** 655–666.
5. Bellamy, R., Ruwende, C., Corrah, T., McAdam, K. P., Whittle, H. C., Hill, A. V. (1998) Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. *N. Engl. J. Med.* **338,** 640–644.
6. Blackwell, J. M., Goswami, T., Evans, C. A., Sibthorpe, D., Papo, N., White, J. K., Searle, S., Miller, E. N., Peacock, C. S., Mohammed, H., Ibrahim, M. (2001) SLC11A1 (formerly NRAMP1) and disease resistance. *Cell Microbiol.* **3,** 773–784.
7. Cellier, M. F., Courville, P., Campion, C. (2007) Nramp1 phagocyte intracellular metal withdrawal defense. *Microbes Infect.* **9,** 1662–1670.
8. Forbes, J. R., Gros, P. (2003) Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane. *Blood* **102,** 1884–1892.
9. Biggs, T. E., Baker, S. T., Botham, M. S., Dhital, A., Barton, C. H., Perry, V. H. (2001) Nramp1 modulates iron homoeostasis in vivo and in vitro: evidence for a role in cellular iron release involving de-acidification of intracellular vesicles. *Eur. J. Immunol.* **31,** 2060–2070.
10. Mulero, V., Searle, S., Blackwell, J. M., 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.
11. Fritsche, G., Nairz, M., Theurl, I., Mair, S., Bellmann-Weiler, R., Barton, H. C., Weiss, G. (2007) Modulation of macrophage iron transport by Nramp1 (Slc11a1). *Immunobiology* **212,** 751–757.
12. Nairz, M., Fritsche, G., Crouch, M-L. V., Barton, H. C., Fang, F. C., Weiss, G. (2009) Slc11a1 limits intracellular growth of *Salmonella enterica* sv. *typhimurium* by promoting macrophage immune effector functions and impairing bacterial iron acquisition. Cell Microbiol **11,** 1365–1381.
13. Zwilling, B. S., Kuhn, D. E., Wikoff, L., Brown, D., Lafuse, W. (1999) Role of iron in Nramp1-mediated inhibition of mycobacterial growth. *Infect. Immun.* **67,** 1386–1392.
14. Soe-Lin, S., Apte, S. S., Andriopoulos Jr., B., Andrews, M. C., Schranzhofer, M., Kahawita, T., Garcia-Santos, D., Ponka, P. (2009) Nramp1 promotes efficient macrophage recycling of iron following erythrophagocytosis in vivo. *Proc. Natl. Acad. Sci. USA* **106,** 5960–5965.
15. Barton, C. H., Whitehead, S. H., Blackwell, J. M. (1995) Nramp transfection transfers Ity/Lsh/Bcg-related pleiotropic effects on macrophage activation: influence on oxidative burst and nitric oxide pathways. *Mol. Med.* **1,** 267–279.
16. Barrera, L. F., Kramnik, I., Skamene, E., Radzioch, D. (1994) Nitrite production by macrophages derived from BCG-resistant and -susceptible congenic mouse strains in response to IFN-γ and infection with BCG. *Immunology* **82,** 457–464.
17. Fritsche, G., Dlaska, M., Barton, H., Theurl, I., Garimorth, K., Weiss, G. (2003) Nramp1 functionality increases inducible nitric oxide synthase transcription via stimulation of IFN regulatory factor 1 expression. *J. Immunol.* **171,** 1994–1998.
18. Fritsche, G., Nairz, M., Werner, E. R., Barton, H. C., Weiss, G. (2008) Nramp1-functionality increases iNOS expression via repression of IL-10 formation. *Eur. J. Immunol.* **38,** 3060–3067.
19. Kjeldsen, L., Cowland, J. B., Borregaard, N. (2000) Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. *Biochim. Biophys. Acta* **1482,** 272–283.
20. Goetz, D. H., Holmes, M. A., Borregaard, N., Bluhm, M. E., Raymond, K. N., Strong, R. K. (2002) The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol. Cell* **10,** 1033–1043.
21. Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., Akira, S., Aderem, A. (2004) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* **432,** 917–921.
22. Holmes, M. A., Paulsene, W., Jide, X., Ratledge, C., Strong, R. K. (2005) Siderocalin (Lcn 2) also binds carboxymycobactins, potentially defending against mycobacterial infections through iron sequestration. *Structure* **13,** 29–41.
23. Wu, H., Santoni-Rugiu, E., Ralfkiaer, E., Porse, B. T., Moser, C., Høiby, N., Borregaard, N., Cowland, J. B. (2010) Lipocalin 2 is protective against *E. coli* pneumonia. *Respir. Res.* **11,** 96.
24. Raffatellu, M., George, M. D., Akiyama, Y., Hornsby, M. J., Nuccio, S.-P., Paixao, T. A., Butler, B. P., Chu, H., Santos, R. L., Berger, T., Mak, T. W., Tsolis, R. M., Bevins, C. L., Solnick, J. V., Dandekar, S., Bäumler, A. J. (2009) Lipocalin-2 resistance confers an advantage to *Salmonella enterica* serotype *typhimurium* for growth and survival in the inflamed intestine. *Cell Host Microbe* **5,** 476–486.
25. Bao, G., Clifton, M., Hoette, T. M., Mori, K., Deng, S-X., Qiu, A., Viltard, M., Williams, D., Paragas, N., Leete, T., Kulkarni, R., Li, X., Lee, B., Kalandadze, A., Ratner, A. J., Pizarro, J. C., Schmidt-Ott, K. M., Landry, D. W., Raymond, K. N., Strong, R. K., Barasch, J. (2010) Iron traffics in circulation bound to a siderocalin (Ngal)-catechol complex. *Nat. Chem. Biol.* **6,** 602–609.
26. Devireddy, L. R., Hart, D. O., Goetz, D. H., Green, M. R. (2010) A mammalian siderophore synthesized by an enzyme with a bacterial homolog involved in enterobactin production. *Cell* **141,** 1006–1017.
27. Nairz, M., Theurl, I., Schroll, A., Theurl, M., Fritsche, G., Lindner, E., Seifert, M., Crouch, M-L. V., Hantke, K., Akira, S., Fang, F. C., Weiss, G. (2009) Absence of functional Hfe protects mice from invasive *Salmonella enterica* serovar *typhimurium* infection via induction of lipocalin-2. *Blood* **114,** 3642–3651.
28. Berger, T., Togawa, A., Duncan, G. S., Elia, A. J., You-Ten, A., Wakeham, A., Fong, H. E. H., Cheung, C. C., Mak, T. W. (2006) Lipocalin

2-deficient mice exhibit increased sensitivity to *Escherichia coli* infection but not to ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. USA* **103,** 1834–1839.
29. Atkinson, P. G., Barton, C. H. (1999) High level expression of Nramp1G169 in RAW264.7 cell transfectants: analysis of intracellular iron transport. *Immunology* **96,** 656–662.
30. Zaharik, M. L., Cullen, V. L., Fung, A. M., Libby, S. J., Kujat Choy, S. L., Coburn, B., Kehres, D. G., Maguire, M. E., Fang, F. C., Finlay, B. B. (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.
31. Crouch, M-L. V., Castor, M., Karlinsey, J. E., Kalhorn, T., 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.
32. Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S. M., Fritsche, G., 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.
33. Ludwiczek, S., Theurl, I., Bahram, S., Schümann, K., Weiss, G. (2005) Regulatory networks for the control of body iron homeostasis and their dysregulation in HFE mediated hemochromatosis. *J. Cell. Physiol.* **204,** 489–499.
34. Nairz, M., Schroll, A., Moschen, A. R., Sonnweber, T., Theurl, M., Theurl, I., Taub, N., Jamnig, C., Neurauter, D., Huber, L. A., Tilg, H., Moser, P. L., Weiss, G. (2011) Erythropoietin contrastingly affects bacterial infection and experimental colitis by inhibiting nuclear factor-κB-inducible immune pathways. *Immunity* **34,** 61–74.
35. Jabado, N., Jankowski, A., Dougaparsad, S., Picard, V., Grinstein, S., 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.
36. Chan, Y. R., Liu, J. S., Pociask, D. A., Zheng, M., Mietzner, T. A., Berger, T., Mak, T. W., Clifton, M. C., Strong, R. K., Ray, P., Kolls, J. K. (2009) Lipocalin 2 is required for pulmonary host defense against *Klebsiella* infection. *J. Immunol.* **182,** 4947–4956.
37. Saiga, H., Nishimura, J., Kuwata, H., Okuyama, M., Matsumoto, S., Sato, S., Matsumoto, M., Akira, S., Yoshikai, Y., Honda, K., Yamamoto, M., Takeda, K. (2008) Lipocalin 2-dependent inhibition of mycobacterial growth in alveolar epithelium. *J. Immunol.* **181,** 8521–8527.
38. Halaas, O., Steigedal, M., Haug, M., Awuh, J. A., Ryan, L., Brech, A., Sato, S., Husebye, H., Cangelosi, G. A., Akira, S., Strong, R. K., Espevik, T., Flo, T. H. (2010) Intracellular *Mycobacterium avium* intersect transferrin in the Rab11(+) recycling endocytic pathway and avoid lipocalin 2 trafficking to the lysosomal pathway. *J. Infect. Dis.* **201,** 783–792.
39. Rodrigues, P. N., Gomes, S. S., Neves, J. V., Gomes-Pereira, S., Correia-Neves, M., Nunes-Alves, C., Stolte, J., Sanchez, M., Appelberg, R., Muckenthaler, M. U., Gomes, M. S. (2011) Mycobacteria-induced anaemia revisited: a molecular approach reveals the involvement of NRAMP1 and lipocalin-2, but not of hepcidin. *Immunobiology* **216,** 1127–1134.
40. Saiga, H., Shimada, Y., Takeda, K. (2011) Innate immune effectors in mycobacterial infection. *Clin. Dev. Immunol.* **2011,** 347594.
41. Sen, C. K., Packer, L. (1996) Antioxidant and redox regulation of gene transcription. *FASEB. J.* **10,** 709–720.

KEY WORDS:
NF-κB · reactive nitrogen species · desferrioxamine · iron · siderophores