Salmonella Utilizes Zinc To Subvert Antimicrobial Host Defense of Macrophages via Modulation of NF-κB Signaling

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ABSTRACT Zinc sequestration by macrophages is considered a crucial host defense strategy against infection by the intracellular bacterium Salmonella enterica serovar Typhimurium. However, the underlying mechanisms remain elusive. In this study, we found that zinc favors pathogen survival within macrophages. Salmonella-hosting macrophages contained higher free zinc levels than did uninfected macrophages and cells that successfully eliminated bacteria, which was paralleled by the impaired production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in bacterium-harboring cells. A profound, zinc-mediated inhibition of NF-κB p65 transcriptional activity affecting the expression of the ROS- and RNS-forming enzymes phos47 and inducible nitric oxide synthase (iNOS) provided a mechanistic explanation for this phenomenon. Macrophages responded to infection by enhancing the expression of zinc-scavenging metallothioneins 1 and 2, whose genetic deletion caused increased free zinc levels, reduced ROS and RNS production, and increased the survival of Salmonella. Our data suggest that Salmonella invasion of macrophages results in a bacterium-driven increase in the intracellular zinc level, which weakens antimicrobial defense and the ability of macrophages to eradicate the pathogen. Thus, limitation of cytoplasmic zinc levels may help to control infection by intracellular bacteria.

KEYWORDS NADPH oxidase, NF-κB, Salmonella, macrophages, nitric oxide synthase, zinc

Macrophages, which constitute the first line of antimicrobial defense, are equipped with a broad repertoire of mechanisms to clear intracellular bacteria (1, 2). One such strategy is to limit the availability of nutrients important for microbial growth, such as iron, copper, manganese, or zinc (3–6). Whereas the role of iron in the host-pathogen interaction in infection by intracellular bacteria such as Salmonella enterica serovar Typhimurium or mycobacteria has been extensively studied (7–9), far less is known about the role of zinc in this setting.

The transition metal zinc (Zn) is essential for a plethora of structural proteins and enzymes and impacts immune cell function and differentiation (10, 11). Zinc deficiency has been linked to impaired B, T, and NK cell responses and inflammatory cytokine production (11). Recent evidence suggests that the accumulation of Zn in the Golgi...
apparatus of activated macrophages triggers the formation of toxic radicals by NADPH oxidase (NOX), thereby contributing to the clearance of Histoplasma capsulatum (12). An excessive cytokine-induced accumulation of Zn and Cu in bacterium-containing phagosomes can intoxicate intracellular pathogens such as mycobacteria (1, 13). Of note, microbes developed measures to circumvent zinc toxicity (4, 14). Hence, both the compartment-specific sequestration of vital metal ions as well as bacterial intoxication by high metal ion concentrations belong to the defense arsenal of the macrophage (4, 15).

On the other hand, most pathogens require zinc for their metabolic needs and for the defense against host-mediated oxidative stress (16). This may be a reason why neutrophils secrete calprotectin, which scavenges zinc and reduces its availability for microbes in the extracellular compartment (4). More evidence for the ambiguous role of zinc in infections comes from randomized clinical trials aiming at improving children’s health by correcting Zn and iron deficiencies. In this setting, dietary zinc and iron supplementation correlated with increased morbidity and mortality from infections (17), part of which may be related to Zn-mediated alterations of the intestinal microbiota (18). However, previous studies also indicated a beneficial effect of Zn supplementation on the incidence and outcome of bacterial infections (11, 19).

Salmonella Typhimurium is a Gram-negative bacterium that resides and replicates within macrophages (20, 21). Several studies have shown that its growth and pathogenicity depend strictly on a sufficient supply of iron (22) and that host mechanisms that restrict iron availability can efficiently control Salmonella proliferation in the cell (23, 24). However, much less is known about the importance of macrophage zinc homeostasis in the control of Salmonella infection. In response to pathogen invasion, macrophages induce numerous antimicrobial pathways, including the formation of reactive oxygen species (ROS) through the NAPDH oxidase complex and reactive nitrogen species (RNS) by inducible NO synthase (iNOS) (2, 25–28). Salmonella can dismantle the radical-producing machinery, for example, by reprogramming host metabolism and gene expression via a type III expression system (T3SS) encoded by Salmonella pathogenicity island 2 (SPI-2) (29, 30). The activation of radical-detoxifying enzymes such as Cu/Zn superoxide dismutase provides another measure to resist oxidative and nitrosative stress, for which zinc is actively acquired from the environment by invading pathogens (31, 32).

Here, we shed light on a previously unrecognized role of zinc in the host-pathogen interplay. We found that Salmonella induces the accumulation of protein-unbound zinc (labile/free zinc) in infected macrophages to impair fully fledged NF-kB activation, which is essential for the transcriptional induction of NADPH oxidase and iNOS. This results in dampened ROS and RNS formation leading to improved pathogen survival. We further demonstrate that free zinc mobilization by genetic deletions of zinc-chelating proteins hinders efficacious antibacterial immune defense.

RESULTS

Salmonella infection induces free zinc accumulation in infected macrophages. We utilized the RAW264.7 macrophage cell line and the intracellular bacterium Salmonella Typhimurium to study the impact of cellular zinc availability on host-pathogen interactions.

Infection of RAW264.7 macrophages with S. Typhimurium resulted in a significant increase in the level of labile intracellular zinc, as measured by FluoZin fluorescence (Fig. 1A). In parallel, the mRNA expression of two important Zn-binding metallothioneins (MTs), MT1 and MT2 (33), was induced, which could be considered a host response mechanism to limit labile zinc levels in cells (Fig. 1B). Of interest, we detected only a minor increase in the total (i.e., free and protein-bound) cellular zinc content following infection using atomic absorption spectrometry (Fig. 1C). This suggests that primarily a shift of zinc from protein-bound sources to the free intracellular zinc pool, rather than the acquisition of the metal from the extracellular space, may occur in macrophages upon Salmonella infection.
Zinc supplementation promotes *Salmonella* infection in macrophages by impairing clearance of the pathogen. To study the biological importance of altered zinc availability for the course of *Salmonella* infection in macrophages, we modulated zinc availability by adding either ZnCl$_2$ or NN$N$,NN$N$-tetakis(2-pyridylmethyl)ethylenediamine (TPEN) (34), which specifically chelates intracellular zinc, to macrophages infected with green fluorescent protein (GFP)-expressing *Salmonella* bacteria (see Fig. S1A in the supplemental material). Neither of the treatments diminished cell viability (Fig. S1B). Interestingly, zinc supplementation significantly elevated the percentage of *Salmonella*-infected macrophages and total numbers of bacteria (Fig. 2A and Fig. S2A). In turn, zinc chelation had an opposite effect on both readouts of infection (Fig. 2A). Of note, the same effects of zinc modulation on the infection rate were observed in mouse primary bone marrow-derived macrophages (Fig. S3). This observation could not be explained by an alteration of the phagocytic uptake of particles such as bacteria or beads, as zinc supplementation decreased rather than increased the phagocytic capacity of macrophages (Fig. S2B and S2C). This led us to hypothesize that the infection-promoting action of increased cellular zinc levels occurs after the entry of the bacteria into the cell. Zinc addition had no effects on bacterial survival and proliferation in *in vitro* cultures, hence excluding the possibility that additional zinc favors extra- or intracellular bacterial growth, resulting in a higher macrophage infection rate (Fig. S4).

To test whether free zinc directly alters intracellular bacterial proliferation, we determined the number of viable bacteria per cell following ZnCl$_2$ or TPEN treatment. Relative numbers of bacteria per cell remained unaffected upon this treatment (Fig. S5A), suggesting that not intracellular pathogen proliferation but rather the susceptibility of macrophages to bacterial invasion is increased upon higher zinc availability.
We then sought to test whether zinc can inhibit the immediate killing of *Salmonella* by macrophages. We thus quantified the percentages of macrophages containing viable and dead bacteria upon zinc modulation and infection with the red fluorescent protein (RFP)-expressing *Salmonella* reporter strain. Intracellular bacteria were additionally stained with an anti-CSA-1 antibody, which recognizes both live and dead *Salmonella* bacteria. Since only metabolically active bacteria produce RFP, we defined viable *Salmonella* bacteria as being CSA-1 positive (CSA-1+/RFP+/H11001) and dead ones as being CSA-1+/RFP− (Fig. S5B). As shown in Fig. 2B, surplus zinc significantly

![Graphs showing the effect of zinc supplementation on infection rate](image)

**FIG 2** Zinc supplementation increases the infection rate by impairing the killing capacities of macrophages. RAW264.7 cells were stimulated with the vehicle, zinc, or TPEN and infected with GFP-expressing (A) and RFP-expressing (B) *S. Typhimurium* strains for the indicated time points. (A) The percentage of *Salmonella*-containing cells among viable macrophages (DAPI+/H11002) was determined by flow cytometry and confirmed by plating of lysed cell cultures. Representative cytometry plots of DAPI− macrophages are presented with a summary graph (*n* = 4). (B) Cells were stained intracellularly with an anti-CSA antibody recognizing both live and killed *Salmonella*. The percentages of macrophages containing RFP+/H11001 live and RFP− dead bacteria among CSA+ cells were determined by flow cytometry. Representative data and a summary graph (*n* = 3) are shown. Statistical significance was assessed by two-way (A) and one-way (B) ANOVAs with Bonferroni post hoc tests. FSC, forward scatter.
reduced the number of macrophages containing dead bacteria, whereas TPEN treatment increased it. These data led us to hypothesize that an increase in the level of free zinc in macrophages may be employed by Salmonella to resist the antimicrobial elimination pathways of the host.

Zinc supplementation affects Salmonella clearance through inhibition of ROS and RNS production. The generation of ROS and RNS by NOX and iNOS in macrophages serves as an efficacious mechanism to eliminate intracellular pathogens (2, 22). These pathways are also of importance in our in vitro infection system, since treatment of macrophages with a ROS scavenger, N-acetyl-cysteine (NAC), or an iNOS inhibitor, N^6-(1-iminoethyl)-L-lysine (L-NIL), increased the numbers of intracellular bacteria (details not shown).

Since elevated free zinc levels hindered the effective clearance of Salmonella, we investigated whether the modulation of zinc concentrations affected ROS and RNS production upon bacterial infection. We detected a drop in nitrite production (the stable end product of the NO pathway) after zinc supplementation (Fig. 3A). The formation of ROS and RNS is tightly controlled by the transcriptional, posttranscriptional, and posttranslational regulation of ROS/RNS-generating enzymes. Upon zinc supplementation, the mRNA levels of iNOS and the key NOX subunit p47phox were significantly reduced (Fig. 3B). Accordingly, a significant reduction of the iNOS protein level was observed as well (Fig. 3C). Of note, treatment with TPEN did not increase p47phox mRNA and iNOS transcript and protein amounts above the control levels, suggesting that the TPEN-mediated improvement of the elimination of bacteria may also involve some other radical-independent antimicrobial pathways.

To test for the possibility that zinc stimulation can protect bacteria against the detrimental reactive oxygen and nitrogen species in a macrophage-independent manner, we monitored the growth of wild-type (WT) Salmonella bacteria in the presence of...
ROS (paraquat) and RNS (S-nitroso-N-acetyl-DL-penicillamine [SNAP]) generators with zinc and TPEN supplementation. As shown in Fig. S6 in the supplemental material, neither the addition of zinc nor its chelation impacted the survival and proliferation of the bacteria under conditions of oxidative and nitrosative stress.

Zinc inhibits NF-κB activation. Nuclear factor kappa B (NF-κB) is a master regulator of iNOS and p47phox mRNA expression (2, 35). Having observed that surplus zinc downregulates both of these transcripts, we asked whether zinc can affect NF-κB transcriptional activity. The phosphorylation and nuclear accumulation of the p65 subunit are surrogates for NF-κB activation. The addition of zinc reduced p65 phosphorylation in infected macrophages, whereas zinc chelation strongly enhanced it (Fig. 4A). Similarly, we observed impaired p65 nuclear trans-localization after zinc addition and the opposite phenomenon after TPEN treatment (Fig. 4B; see also Fig. S7 in the supplemental material). To further corroborate the impact of zinc on the transcriptional activity of p65 NF-κB, we performed a chromatin immunoprecipitation assay with a phospho-p65 antibody. As shown in Fig. S5A in the supplemental material, zinc almost completely abrogated p65 binding to the proximal site in the iNos promoter after Salmonella infection as well as to promoters of other classical p65 target genes, Icam and Cxcl2. Zinc chelation in turn increased the affinity of p65 for both binding sites in

**FIG 4** Increases in free zinc concentrations hamper NF-κB signaling. RAW264.7 cells were stimulated with the vehicle, zinc, or TPEN and infected with S. Typhimurium for the indicated times. (A) Levels of pSer536 NF-κB and total NF-κB in whole-cell lysates were measured by Western blotting. Actin served as a loading control. Representative results of one experiment are shown (n = 2). (B) The immunofluorescent NF-κB signal intensity in the nucleus and cytoplasm was measured by fluorescence microscopy. Representative images originating from one representative experiment are shown (n = 2). Data presented in the graph refer to signal intensities in at least 10 high-power fields under each experimental condition. Statistical significance was calculated by one-way ANOVA with a Bonferroni post hoc test.
the iNos promoter region (Fig. S8A). Our finding that zinc supplementation impairs p65 transcriptional activity in general is further supported by the effects of surplus zinc on mRNA levels of the NF-κB-regulated cytokine genes Il1b, Il6, Il10, and Tnf (Fig. S8B). Notably, these effects were specific for the NF-κB pathway, since the activity of extracellular signal-regulated kinase (ERK), STAT1, and STAT3 signaling remained unchanged (Fig. S9).

Taken together, these data show that the expansion of cellular zinc levels impairs the killing capacity of macrophages by inhibiting NF-κB activation and the downstream transcriptional activation of iNOS, phox47, and several proinflammatory cytokines.

**Salmonella uses free zinc to overcome host clearance.** As an intracellular pathogen, *Salmonella* developed a plethora of strategies to bypass antimicrobial effector pathways of macrophages. We hypothesized that the cellular accumulation of free cytoplasmic zinc causing the inhibition of ROS/RNS formation may pose such a bacterium-driven protective mechanism.

To test this, we infected macrophages with RFP reporter-expressing *Salmonella* bacteria and sorted bacterium-hosting (RFP+/H11001) and bacterium-cleared/uninfected (RFP-/H11002) macrophages from these cultures. Macrophages containing viable *Salmonella* bacteria accumulated significantly more zinc (Fig. 5A) and expressed more zinc-sensitive Mt1 and Mt2 transcripts (Fig. 5B) than did *Salmonella*-naive or *Salmonella*-clearing macrophages. As with zinc supplementation, we also observed significantly reduced ROS production (Fig. 5C) and iNOS mRNA (Fig. 5D) and iNOS protein (Fig. 5E) expression levels in *Salmonella*-hosting macrophages compared to bacterium-negative cells. Importantly, this was paralleled by a profound downregulation of phosphorylated p65 and an inhibition of the downstream p38 pathway in RFP+ macrophages compared to *Salmonella*-clearing ones (Fig. 5F). No such consistent regulatory pattern was seen among other signaling pathways involved in antibacterial activity, such as STAT1, STAT3, and ERK (see Fig. S10 in the supplemental material). These data support the hypothesis that either increased zinc levels in macrophage are a predisposing factor for *Salmonella* infection or *Salmonella* invasion of macrophages may promote cytoplasmic zinc accumulation, which impairs the immediate NF-κB-mediated antimicrobial host defense.

**Mt1/2 knockout in macrophages favors intracellular Salmonella survival.** The zinc-binding metallothioneins MT1 and MT2 are vital regulators of cellular free zinc levels. Their expression is induced upon *Salmonella* infection and specifically increased in *Salmonella*-hosting macrophages. We hypothesized that the upregulation of MT1 and MT2 may pose a host response mechanism to limit zinc availability for intracellular bacteria. To study their specific role in our infection model, we disrupted the Mt1 and Mt2 genes in macrophages using the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technique (see Fig. S11A in the supplemental material). This event results in a boost of cellular free zinc levels, even without zinc supplementation, in resting and infected cells (Fig. S11B and S11C). Comparably to zinc supplementation, Mt knockout cells displayed increased *Salmonella* colonization (Fig. 6A and Fig. S11D), reduced ROS levels (Fig. 6B), and impaired RNS production as assessed by nitrite and iNOS protein levels (Fig. 6C) compared with wild-type macrophages. These results further corroborate the key impact of labile intracellular zinc on intracellular bacterial survival by modulating antimicrobial ROS and RNS formation in *Salmonella*-infected macrophages. In addition, we identify infection-driven MT1 and MT2 expression with the subsequent reduction of intracellular zinc levels as an important antibacterial host response mechanism.

**DISCUSSION**

The influence of infection-induced alterations of zinc homeostasis on the course of inflammatory processes and the efficacy of bacterial clearance is largely unknown. In this study, we examined the role of zinc availability in the control of infection by the intracellular bacterium *Salmonella* Typhimurium and its impact on macrophage-mediated antimicrobial effector pathways. Both the excessive accumulation of zinc in...
pathogen-containing cell compartments as well as its restriction from invading pathogens were previously described to constrain the growth of various intra- and extracellular microbes (1, 4, 11–14, 16–19).

Here, we observed that *Salmonella*-infected macrophages display higher free zinc levels than do cells unexposed to the pathogen or macrophages that successfully eliminated bacteria. However, the rise in the amount of total cellular zinc was marginal, suggesting a shift from protein-bound zinc to the free-ion compartment rather than the active uptake of the metal into cells. To our surprise, zinc supplementation promoted neither bacterial intoxication nor clearance but instead increased the vulnerability of

FIG 5 Zinc accumulation can pose a pathogen’s strategy to evade clearance in macrophages. RAW264.7 cells were infected with RFP-expressing *S. Typhimurium* for 6 h. Viable cells (DAPI−) containing live *Salmonella* (Stm−) and no/dead *Salmonella* (Stm+) bacteria were analyzed by flow cytometry and sorted by using a fluorescence-activated cell sorter. (A) Free zinc levels were determined by Fluozin staining. Representative Fluozin signal histograms are shown. The plot depicts the ΔMFI (n = 3). (B) Mt1 and Mt2 gene expression levels measured by qRT-PCR in fluorescence-activated cell sorter-sorted DAPI− *S. Typhimurium*-positive and DAPI− *S. Typhimurium*-negative macrophages (n = 3). (C) ROS production was measured by CellROX staining. The plot depicts the ΔMFI (n = 3). (D) Nos2 gene expression was measured by qRT-PCR in fluorescence-activated cell sorter-sorted viable *S. Typhimurium*-positive and *S. Typhimurium*-negative macrophages (n = 3). (E) Percentages of cells positive for the intracellular iNOS protein were determined by flow cytometry. Representative cytometry plots are shown with a summary graph (n = 3). (F) pSer536 NF-κB, total NF-κB, phospho-p38, and total p38 levels were measured in fluorescence-activated cell sorter-sorted DAPI− *S. Typhimurium*-positive and DAPI− *S. Typhimurium*-negative macrophages by Western blotting. Results of a representative experiment are presented (n = 2). Statistical significance was assessed by two-way ANOVA with a Bonferroni post hoc test.
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December 2017 Volume 85 Issue 12 e00418-17 iai.asm.org

RAW264.7 as well as murine primary bone marrow-derived macrophages to infection. This was directly linked to the reduced formation of antibacterial ROS and RNS in zinc-supplemented macrophages (2, 36, 37). Free zinc inhibited p65 NF-κB activation, hence dampening the transcriptional expression of the ROS- and RNS-generating enzymes p47phox and iNOS (38, 39). This is in agreement with the previously described inhibitory effect of zinc on other NF-κB-driven effector pathways (40–43), which impacted the immune response in polymicrobial sepsis (44). Therefore, zinc was proposed to upregulate the NF-κB inhibitor A20 and to bind directly to IκB kinase beta.
strains were described previously (49, 50).

The ATCC (ATCC 14028) Reporter GFP-expressing (ST-GFP) and RFP-expressing (ST-RFP) Salmonella
infection by intracellular bacteria such as study the functional activity of MT proteins in these settings.
points to the need to not only evaluate MT expression dynamically over time but also
MT2 transcripts within macrophages containing either viable or dead bacteria, which
phages blunts NF-
dismutase (SOD) (31, 32), which also protect microbes from oxidative damage by
host-generated radicals. However, this possibility seems unlikely, since zinc supplementation
did not modify bacterial growth in the presence of ROS and RNS generators in
macrophage-free setting.

We thus propose that the accumulation of free zinc in macrophages serves as a
protective mechanism enabling bacteria to withstand killing by macrophages (10, 26).
Using a fluorescent reporter-expressing Salmonella strain, we found that macrophages
hosting viable bacteria accumulate dramatically higher free zinc levels than do cells
that cleared pathogens, which was paralleled by the downregulation of ROS and RNS
formation. However, the underlying mechanism for cellular Zn mobilization remains
elusive. Nevertheless, Salmonella may use the increased free zinc levels for its own
purposes, such as dismantling the killing machinery. Our data obtained with the Mt1/2
knockout cell lines not only underline the crucial role of free zinc in infected cells but
also stress the vital contribution of metallothioneins to the inhibition of bacterial
colonization (47). The robust induction of MT1 and MT2 by zinc supplementation,
inflammatory stimuli, or bacterial products may be seen as a defense strategy of
macrophages to reduce the concentration of free zinc ions in the cell and hence enable
the fully fledged NF-κB-mediated cytokine response and bacterial clearance (41, 48).
However, there were only nonsignificant differences in the expression levels of MT1 and
MT2 transcripts within macrophages containing either viable or dead bacteria, which
points to the need to not only evaluate MT expression dynamically over time but also
study the functional activity of MT proteins in these settings.

In summary, we propose a novel role for labile cytoplasmic zinc in the course of
infection by intracellular bacteria such as Salmonella. Zinc accumulation in macro-
phages blunts NF-κB activation and impairs NF-κB-dependent bacterial clearance
mediated by iNOS and p47phox. This phenomenon can also be regarded as a strategy
of Salmonella and possibly other intracellular bacteria to overcome oxidative and
nitrosative stress in macrophages. Therapeutic strategies that reduce zinc accumulation
in cells or that increase the zinc-scavenging capacity of metallothioneins may prove
effective for the treatment of infections by intracellular microbes.

### MATERIALS AND METHODS

**Bacterial strains.** Wild-type Salmonella enterica serovar Typhimurium bacteria were obtained from the ATCC (ATCC 14028). Reporter GFP-expressing (ST-GFP) and RFP-expressing (ST-RFP) Salmonella
strains were described previously (49, 50).

**Cell culture and Salmonella infection in vitro.** The murine macrophage cell line RAW264.7 was kept in Dulbecco’s modified Eagle’s medium (DMEM) with 2 mM l-glutamine and 10% fetal calf serum (FCS)
(Biochrom). Cells were routinely collected by scraping. Cells were seeded at a density of 5 × 10⁴ cells/cm²
a day before infection. Infections with WT, ST-GFP, and ST-RFP Salmonella bacteria were performed at a
multiplicity of infection (MOI) of 1:10, as described previously (23), 1 h after bacterial challenge medium
was changed to a gentamicin-containing one (25 μg/ml). A total of 100 μM ZnCl₂ or 5 μM TPEN (Sigma)
was added to the cultures concomitantly with the bacteria and was present for the whole infection
period.

**Flow cytometry, cell sorting, and fluorescence microscopy.** Flow cytometry measurements were
performed with a Beckman-Coulter Gallios device and analyzed with FlowJo software (FlowJo LLC). ΔMFI
is defined as the difference in the signal intensity between an unstained control and a stained sample.
For sorting, a FACSAria I device (Becton Dickinson) was used. Microscopy images were acquired with an
Axioskop fluorescence microscope (Zeiss) and analyzed with ImageJ software.

**Determination of free and total zinc.** Macrophage cultures were incubated with 5 μM Fluozin 3-AM
(Life Technologies) for 1 h at 37°C. Subsequently, medium was removed, and cells were incubated in
phosphate-buffered saline (PBS) for 30 min at 37°C. The green fluorescence of the probe was detected
by fluorescence microscopy and flow cytometry. Total zinc concentrations were measured by atomic
absorption spectrometry as described previously (51).

**Quantitative real-time PCR and Western blotting.** RNA was isolated and reverse transcribed as
described previously (52). Relative gene expression was calculated with the ΔΔCt method, normalizing
the results to the value for the Hprt gene. The list of primer oligonucleotide sequences can be found in
Table S1 in the supplemental material. Protein extraction and Western blotting were performed as

(IKKB), hence moderating its activity (45, 46). We further showed that Zn diminishes p38
and p65 phosphorylation and, consequently, NF-κB transcriptional activity, hence
blunting antimicrobial host responses.

Also, Zn may act as a cofactor of antioxidative enzymes such as Zn/Cu superoxide
dismutase (SOD) (31, 32), which also protect microbes from oxidative damage by
host-generated radicals. However, this possibility seems unlikely, since zinc supplementation
may use the increased free zinc levels for its own
purposes, such as dismantling the killing machinery. Our data obtained with the Mt1/2
knockout cell lines not only underline the crucial role of free zinc in infected cells but
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colonization (47). The robust induction of MT1 and MT2 by zinc supplementation,
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However, there were only nonsignificant differences in the expression levels of MT1 and
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mediated by iNOS and p47phox. This phenomenon can also be regarded as a strategy
of Salmonella and possibly other intracellular bacteria to overcome oxidative and
nitrosative stress in macrophages. Therapeutic strategies that reduce zinc accumulation
in cells or that increase the zinc-scavenging capacity of metallothioneins may prove
effective for the treatment of infections by intracellular microbes.
described previously (52), using primary rabbit antibodies against NF-κB p65 (catalog no. 4764), pSer536–NF-κB p65 (catalog no. 3033), p38 (catalog no. 9212), phosphorylated p38 (p-p38) (catalog no. 4511), ERK (catalog no. 4696), p-ERK (catalog no. 4370), STAT1 (catalog no. 9172), p-STAT1 (catalog no. 9167), STAT3 (catalog no. 9132), p-STAT3 (catalog no. 9134), and actin (catalog no. A2066) (all from Cell Signaling Technology, except for actin, which was purchased from Sigma-Aldrich). The dilution of primary antibodies was 1:1,000. As a secondary antibody, a goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (catalog number P0448; Dako) was used (1:2,000).

**Intracellular flow cytometry staining and **Salmonella** killing assay.** Macrophages infected with RFP-expressing *Salmonella* bacteria were harvested and fixed with 4% formalin in PBS for 15 min at room temperature, washed twice with PBS, and blocked in blocking buffer (5% horse serum and 0.3% Triton X-100 in PBS) at room temperature for 15 min at room temperature, washed twice with PBS, and blocked in blocking buffer (5% horse serum and 0.3% Triton X-100 in PBS) at room temperature for 15 min. For intracellular iNOS staining, cells were incubated for 1 h with a 1:200 dilution of primary antibody against NF-κB p65 (catalog no. 4764; Cell Signaling Technologies), diluted in antibody dilution buffer (1% bovine serum albumin [BSA] and 0.3% Triton X-100 in PBS) at 4°C overnight. Next, staining with a 1:200 dilution of Dylight488 donkey anti-rabbit IgG (BioLegend) for 1 h was performed, followed by 3 PBS wash steps and mounting with 4',6-diamidino-2-phenylindole (DAPI)-containing fluorescence mounting medium (Dako). Cytoplasmic and nuclear NF-κB mean fluorescence intensities were measured by using ImageJ software (see Fig. S4 in the supplemental material).

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**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/IAI.00418-17](https://doi.org/10.1128/IAI.00418-17).

**SUPPLEMENTAL FILE 1, PDF file; 6.0 MB.**

**ACKNOWLEDGMENTS**

We declare no financial conflict of interest.

This work was supported by Chinese research funds (project 31472118 to K.Z.), a research grant from the China Scholarship Council (CSC) (grant no. 201406910047 to A.W.), Austrian research funds (project TRP-188 to G.W.), the FWF doctoral program W1253-HOROS (to G.W. and C.L.-F.), and a research grant from the Chinese Ministry of Science (to A.W.).
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Correction for Wu et al., “Salmonella Utilizes Zinc To Subvert Antimicrobial Host Defense of Macrophages via Modulation of NF-κB Signaling”

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Volume 85, no. 12, e00418-17, 2017, https://doi.org/10.1128/IAI.00418-17. Page 11, line 58, Acknowledgments: “Austrian research funds (project TRP-188 to G.W.)” should read “Austrian research funds (project TRP-188 to G.W. and P-28302 to I.T.).”

Citation Wu A, Tymoszuk P, Haschka D, Heeke S, Dichtl S, Petzer V, Seifert M, Hilbe R, Sopper S, Talasz H, Bumann D, Lass-Flörl C, Theurl I, Zhang K, Weiss G. 2018. Correction for Wu et al., “Salmonella utilizes zinc to subvert antimicrobial host defense of macrophages via modulation of NF-κB signaling.” Infect Immun 86:e00881-17. https://doi.org/10.1128/IAI.00881-17.

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