Zinc dyshomeostasis during polymicrobial sepsis in mice involves zinc transporter Zip14 and can be overcome by zinc supplementation

Inga Wessels, and Robert J. Cousins*

Food Science and Human Nutrition Department
Center for Nutritional Sciences
College of Agricultural and Life Sciences
University of Florida
Gainesville, Florida

Corresponding Author:
Robert J. Cousins
352-392-2133
cousins@ufl.edu

Other Authors:
Inga Wessels
inga.wessels@gmx.net

Running head: Zip14 transporter influences murine polymicrobial sepsis
Abstract

Integrity of the immune system is particularly dependent on the availability of zinc. Recent data suggest that zinc is involved in the development of sepsis, a life-threatening systemic inflammation with high death rates, but with limited therapeutic options. Altered cell zinc transport mechanisms could contribute to the inflammatory effects of sepsis. In this regard, Zip14, a zinc importer induced by pro-inflammatory stimuli, could influence zinc metabolism during sepsis and serve as a target for therapy.

Using cecal ligation and puncture (CLP) to model polymicrobial sepsis, we narrowed the function of ZIP14 in regulating zinc homeostasis to hepatocytes, while hepatic leukocytes were mostly responsible for driving inflammation expressing higher IL-1β, TNFα, S100A8 and MMP-8. Using Zip14 knockout (KO) mice as a novel approach, we found that ablation of Zip14 produced a delay in development of leukocytosis, prevented liver zinc accumulation, altered the kinetics of hypozincemia and drastically increased serum IL-6, TNFα, and IL-10 concentrations following CLP. Hence this model revealed that the zinc transporter ZIP14 is a component of the pathway for zinc redistribution that contributes to zinc dyshomeostasis during polymicrobial sepsis. In contrast, using the identical CLP model, supplemental dietary zinc reduced sepsis severity as cytokines, calprotectins, and blood bacterial loads were ameliorated. We conclude that the zinc transporter ZIP14 influences aspects of the pathophysiology of nonlethal polymicrobial murine sepsis induced by CLP through zinc delivery. The results are promising for the use of zinc and its transporters as targets for future sepsis therapy.

Keywords: Zinc transport, sepsis, zinc metabolism, cytokines.
Sepsis is one of the leading inflammatory diseases, causing millions of deaths annually and producing immense costs for health systems worldwide (22, 28). In spite of intense research, the mechanisms underlying sepsis are not completely understood and treatment is limited to symptomatic approaches with limited success (44). An association between altered zinc homeostasis and the severity of the inflammatory reactions has been suggested by several studies using various disease models and systems. These include studies of LPS-induced endotoxemia, acute stress and sepsis in pigs (8, 27), fowl (26, 38), rodents (14, 15, 40) and human subjects (17).

The acute response to endotoxin in vivo includes hypozincemia and altered kinetics of zinc distribution to specific tissues (8). Such changes in body zinc redistribution are believed to be controlled through selective regulation of zinc transport pathways. Two zinc transporter families comprised of 24 members (ZnT family with 10 and Zip family with 14), exhibiting differential modes of regulation and cell-type expression, alter zinc metabolism to meet dietary and physiologic needs (29). Using our previous experiments on the responsiveness of zinc homeostasis to cytokine/hormonal stimuli as a basis, we hypothesized that microbial attack would drastically alter expression of specific Zn/Zip transporters in mice. Earlier, Begum et al. (7) showed that LPS induced a novel gene in monocytes that was subsequently identified as Zip8. Experiments focused on liver using a qPCR screen of individual transporter gene transcripts showed that Zip14 was the most highly induced transporter in liver of mice treated with LPS to induce endotoxemia (31). Subsequently, Zip14 has been documented to be expressed in multiple tissues of mice under a variety of physiological
conditions including endotoxemia (2, 3, 30). The liver has been a prime target of these investigations. Liver dysfunction is a major factor that contributes to the severity of sepsis (6, 35). This often fatal condition is accompanied by endotoxemia and drastic changes in cytokine production and secretion (1, 13, 23, 47).

We hypothesized based on the responsiveness of Zip14 expression to endotoxin, that this zinc transporter would be induced in liver during sepsis and hence would contribute to the altered zinc homeostasis and functional outcomes observed under such conditions. Using Zip14 knockout (KO) mice as a novel approach, we report that the zinc transporter ZIP14 is a component of the pathway for zinc that contributes to zinc dyshomeostasis during polymicrobial sepsis. Our experiments demonstrate ZIP14 is involved in the response to sepsis and that supplemental dietary zinc has beneficial anti-inflammatory effects during sepsis.

MATERIAL AND METHODS

Animals and Diets. Mice used were of the C57BL/6 strain. Genotypes were either Zip14+/+ (WT) or Zip14-- (KO). Derivation and characterization of mice of the KO genotype has been described previously (2, 3). Both male and female mice were used at 8-20 weeks of age. Only one gender was used to generate a specific data set. The mice were fed a commercial rodent diet (Harlan Teklad 7912), except for one series in which either a normal or supplemental zinc diet was fed. Those diets contained 30 mg Zn/kg or 180 mg Zn/kg, respectively as described previously (3). Mice were euthanized by exsanguination via cardiac puncture up to 72h after CLP or SHAM operation. Isoflurane anesthetic was used for all procedures. Buprenorphine (0.1 mg/kg, sc) was...
used for analgesia as needed. Protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

*Cecal Ligation and Puncture.* Polymicrobial sepsis was induced by cecal ligation and puncture (CLP) using established methodology (11). Briefly, laparotomy was performed under anesthesia, the cecum was exteriorized and ligated 1 cm from the distal end. After puncturing the cecum once through the cecum with a syringe needle (27 gauge), the cecum was returned and the surgical site was closed with staples. For SHAM operation, laparotomy and exteriorization of the cecum were performed, but without ligation or puncture. No mortality was observed using the 27 gauge needle for the puncture.

**Biochemical Analyses.** TNFα and IL-10 levels were measured by enzyme-linked immunosorbent assay (Ebioscience, San Diego, CA). IL-6, MMP-9, S100A8, S100A9 levels were quantified by using a customized magnetic multiplexing assay (R & D Systems, MN, USA). Plasma ALT level was measured by a colorimetric end point method as described previously (3). Serum was obtained from blood by centrifugation. For white blood cell (WBC) generation, coagulation was inhibited by adding EDTA to the blood. Blood erythrocytes were lysed using lysis buffer (0.15 M ammonium chloride, 10 mM sodium bicarbonate, pH 7.4). WBC were isolated from the buffy coat and were washed with PBS twice WBC viability was >95%. Zinc concentrations of serum and tissues were measured by flame atomic absorption spectrophotometry (AAS) and were normalized for tissue weight. Tissue was digested in HNO₃ prior to assay by AAS. Non-heme iron was measured colorimetrically (37).
Isolation of leukocytes and parenchymal cells from liver. The mice were anesthetized and the abdomen was wiped with 70% ethanol. The outer skin of the peritoneum was cut to expose the peritoneal cavity. After exsanguination by cardiac puncture, the gall bladder was removed and inferior vena cava was severed. The liver was perfused by injecting 10 ml of ice cold PBS into the hepatic portal vein using a 27G needle. Afterwards, both liver and spleen were removed and placed into ice cold FACS buffer (PBS, 5% BSA). Single-cell suspensions from the liver were generated by forcing the tissue through a 70 μm cell strainer using the plunger of a 5 ml syringe. An aliquot of this cell suspension was saved for analyses of bacterial load and the remaining cells were washed twice with ice-cold FACS buffer. The cell pellet was resuspended in 20 ml of isotonic Percoll (33.75%) at room temperature and centrifuged at 700 x g for 12 min. Hepatocytes, designated as HP, were collected as a disc like sheet floating on top of the Percoll gradient and were washed in wash buffer (William’s Medium E; 10 mM HEPES, pH 7.3) and used to generate RNA for expression analysis. The leukocyte-containing pellet was suspended in 4 ml of Tris-buffered Ammonium Chloride (TAC) buffer (17 mM Tris, 140 mM NH₄Cl) for 10 min., under layered with 1 ml FCS/EDTA (10 mM EDTA) and centrifuged again. After one more washing step, the cells were suspended in FACS/EDTA buffer (FACS buffer; 5 mM EDTA) for further analyses and were designated the HL fraction. The spleen was passed through a cell strainer using the plunger as above. An aliquot of the homogenate was used to analyze bacterial load. These methods were adapted from procedures designed by Wang et al. (43). To measure viability, cells were stained with propidium iodide (Sigma-Aldrich) and
analyzed using flow cytometry as described before (19). Viability averaged 90% for both HP and HL populations.

*Immunoblotting.* Polyclonal rabbit antibody against Zip14 were raised in-house as described previously (31). The rabbit IgG fractions were affinity-purified. Liver tissue samples were flash frozen in liquid nitrogen at collection. Frozen liver tissue was homogenized in lysis buffer (20 mM TrisHCl, 1% Triton X-100, 10% Glycerol, 137 mM NaCl, 2 mM EDTA) containing protease inhibitor cocktail (Santa Cruz Biotechnology Santa Cruz, CA, USA) and sodium vanadate as a phosphatase inhibitor. Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Transfer to nitrocellulose membrane was confirmed by Ponceau Red staining. Immunoreactivity was visualized by enhanced chemiluminescence.

*RNA Isolation and Quantitative Polymerase Chain Reaction.* Liver tissue was collected in RNAlater (Qiagen, Austin, TX, USA) and homogenized in TRIzol reagent (Ambion) using a Bullet Blender® (Next Advance, NY, USA). Cells isolated from liver and blood were placed directly in TRIzol reagent and RNA was isolated according to the manufacturer’s protocol. One µg of RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) and diluted 1:40 for PCR. For analyses of Zip4, Zip6, Zip8, Zip10, PCNA, Hepcidin and ZnT1 mRNAs primers and probes were used together with TaqMan Reagents (Life Technologies). All other genes were analyzed using Primers together with SYBR Green reagent (Life Technologies). Relative quantitation were calculated using a standard curve and normalized using TATA binding protein (TBP) mRNA (2, 3).
Measurement of free intracellular zinc with FluoZin-3AM. Free zinc was measured as described previously for calcium (19) using FluoZin-3 AM ester (1 μM, Invitrogen). The zinc-dependent fluorescence was analyzed with Accuri C6 (BD Bioscience, Franklin Lakes, NJ, USA) using Accuri C6 Software. The concentration of intracellular labile zinc was calculated from the mean fluorescence with the formula $[\text{Zn}] = K_D \times \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}$ using a dissociation constant for the Zn/FluoZin-3AM complex of 8.9 nM and determining the maximal and minimal fluorescence by addition of zinc (100 μM) and pyrithione (50 μM) or TPEN (50 μM), respectively.

Measurement of bacterial load. Fifty μl of each sample (whole blood, liver homogenate, spleen homogenate) were spread onto pre-dried tryptic soy agar plates containing 10% sheep blood. Plates were incubated at 37°C overnight and the number of CFUs was counted.

Statistics. Statistical significance of experimental results was analyzed using GraphPad Prism software version 5 (GraphPad software, La Jolla, CA, USA). For single comparisons, *$P<.05$, **$P<.01$ and ***$P<.001$ are used for data significantly different from the SHAM control as were determined by Student’s $t$ test. For multiple comparisons, significant differences at $P<.05$, determined by ANOVA/Tukey’s test, and indicated by different letters or by ANOVA/Bonferroni test to compare Zip14KO mice with WT mice and to test the effect of high and low dietary zinc and indicated by *$P<.05$, **$P<.01$, ***$P<.001$.

RESULTS

Acute sepsis alters zinc homeostasis and coincides with activation of white blood cells. Cecal ligation and puncture (CLP) using a 27-gauge needle produced a septic
response that included serum hypozincemia and liver zinc accumulation compared to SHAM-operated mice (Fig. 1A). White blood cell (WBC) counts during CLP were inversely proportional to the reduction in serum zinc concentration suggesting a relationship may exist for these processes (Fig. 1A). No significant changes in cell counts in the spleen were observed (data not shown). Induction of liver metallothionein-1 (MT-1) mRNA was evident in mice after CLP with increases to nine fold compared to SHAM animals after 72h (Fig. 1A). MT-1 up-regulation is indicative of increased cellular zinc accumulation. Increased zinc levels in other organs including the spleen (data not shown) were detected but were negligible in magnitude compared to the liver.

Interestingly, there was a burst of MT-1 mRNA abundance in the WBC population 3-9h after CLP (Fig. 1A). This could result from an influx of intracellular zinc. Labile zinc-levels as assessed via FluoZin3 peaked 3h after CLP and then decreased significantly compared to SHAM mice.

To more deeply explore the events that are congruent with the transient zinc redistribution following CLP, we monitored the expression of inflammatory mediators and anti-microbial peptides by liver (Fig. 1B) within the first 24h following CLP. Liver IL-6 and transcripts for both components of the calprotectin heterodimer (S100A8 and S100A9) in liver of these mice underwent CLP surgery was markedly increased (Fig. 1B). In contrast, WBC showed modest changes in IL-6, TNFα, and S100A8/9 mRNAs (data not shown). Of note liver showed significant increases in IL-10 mRNA and MMP-9 transcripts. These responses are considered as anti-inflammatory and anti-microbial, respectively.
Results obtained for mRNA expression were comparable to significant increases of serum IL-6, MMP-9 and S100A9 proteins following CLP (Fig. 1C). Elevated levels of S100A8, TNFα and IL-10 were also detected in the serum of CLP mice, but did not reach statistical significance (data not shown). Protein levels peaked between 3h and 24h after CLP for all mediators measured, returning to basal levels after 48h. Results underline the differential contribution of the liver and WBC to the inflammatory response and levels of serum mediators during sepsis.

The response of parameters (tissue and serum zinc, MT-1 expression) examined following CLP with a single puncture using a 25-gauge needle was greater (data not shown). Some mortality was observed with the use of the 25-gauge needle. Hence, a single puncture with the 27-gauge needle, where no mortality was observed, was used in all of the experiments reported.

*Zip14 expression in liver is strongly elevated throughout the duration of sepsis.* CLP induced a transient wave of changes in zinc transporter mRNAs in liver. Of all 14 Zips and 10 ZnTs tested, maximal increases for Zip4, Zip6, Zip10 and Zip14 mRNAs were detected at 1h, 9h and 24h post CLP, respectively (Fig. 2A). Changes in liver Zip8 mRNA were minimal over the entire 72h period post-CLP. The increases in Zip4, Zip6 and Zip10 expression were transient and returned to the near normal levels by 72h. In contrast, Zip14 mRNA increased by 9h after CLP surgery and was the only transporter mRNA that was constantly elevated in CLP animals compared to SHAM animals throughout the 72h period after CLP (Fig. 2A). Western blots confirmed up-regulation for ZIP14 protein (Fig. 2B). Expression of the other transporters only slightly changed at the
protein level (data not shown). Therefore, these data suggest ZIP14 is up-regulated to sustain hepatic zinc transport during sepsis.

Differences in zinc homeostasis, cytokines and host defensive mediators during sepsis involve hepatocytes and liver leukocytes. The observation of a sequential induction of zinc transporter and cytokine transcripts in total liver RNA extracts raises the question about the contributions of individual cell types. To answer this question, hepatocytes (parenchymal cells, HP) and hepatic leukocytes (HL) were separated and analyzed for relative expression of transcripts for markers of zinc homeostasis, cytokines and host defensive factors. Purity of the isolated cell populations was established through expression of the lineage markers hepcidin (HP) and F4/80 (HL) as assessed using qPCR (Fig. 3A). Viability was confirmed using propidium iodine staining and was approximately 90% (data not shown). Higher expression of MT-1 and Zip14 mRNAs and a higher intracellular labile zinc content were found in HP compared to HL (Fig. 3B). This demonstrates, along with the increase in total liver zinc concentrations after CLP (Fig. 1A), the significance of the HP in zinc-redistribution to the liver during sepsis.

While Zip4 mRNA appeared to be primarily expressed by HP, Zip6 mRNA expression was restricted to HL. Both cell types expressed Zip10 following CLP (Fig. 3C). Transcripts for the inflammatory markers, TNFα, IL-1β and S100A8 were elevated in HL by 9h after CLP. IL-10 mRNA expression by HP was initially high but by 9h levels were comparable in HL. IL-6 was expressed by both cell types (Fig. 3D). MMP-8 mRNA expression was examined and found to be more highly expressed in HL than in HP after CLP. These data suggest that in this in vivo model of polymicrobial sepsis, HP functions
to regulate zinc homeostasis, but also contributes to inflammation via IL-6 and IL-10 production.

Zinc homeostasis in response to sepsis is significantly changed in Zip14 KO mice. In order to examine the potential advantage of zinc redistribution during sepsis, the availability of the Zip14- knockout mouse strain allowed us to examine the role that ZIP14 mediated zinc re-distribution plays in the response to CLP-induced sepsis. The response of the serum zinc concentration in the KO mice following CLP was different than that observed in the WT mice. Specifically, the serum zinc concentration was only significantly depressed (P < .01) at 24h after CLP in the KO mice (Fig. 4A). The delayed response observed in mice when ZIP14 is not produced suggests that the initial hypozincemia of polymicrobial sepsis is produced by a compensatory mechanism involving other zinc transporters (Fig. 4A). In support of that hypothesis is that Zip4, Zip6 and Zip10 mRNAs are significantly elevated in the KO mice compared to the WT at 24h following CLP (Fig. 4A). The increase in WBC counts of the WT mice was inversely related to the extent of hypozincemia. The WBC counts from blood of the KO mice did not closely follow the serum zinc levels, however (Fig. 4A). This suggests that hypozincemia influences the level of circulatory WBC. In contrast, ablation of Zip14 prevented the accumulation of total liver zinc found in the WT mice after CLP (Fig. 4B). Of note is that the Zip14 ablation caused an induction of MT-1 mRNA following CLP suggesting that either the KO mice retained a pool of zinc that activated the MTF-1 transcription factor or that the induction is mediated by cytokines, e.g. the increase detected in circulating IL-6. The demonstrated necessity of ZIP14 for hepatic zinc accumulation (Fig. 4A) suggests that ZIP14 may function in intracellular
processing/utilization of zinc as well as uptake at the cell surface. Surprisingly, hepcidin mRNA expression was significantly stronger in KO animals after CLP than in WT animals (Fig. 4C). The high hepcidin mRNA levels in the KO mice, perhaps a reflection of elevated IL-6, are consistent with the similar levels of NHI in liver of both genotypes (Fig. 4C). Apoptosis of liver cells, based on the marker PD-L1 mRNA, was significantly increased in the septic KO animals and was decreased in WBC (Fig. 4C). On the other hand, liver cell proliferation, based on PCNA mRNA, was significantly decreased in the liver of KO animals. It was lower in WBC as well, but not significantly.

Knockout of Zip14 results in altered cytokine production but not sepsis progression. Western analysis proved that ZIP14 is not produced in the liver of the KO mice in response to CLP. In contrast cell activation, indicated by STAT3 phosphorylation, was comparably enhanced in both genotypes (Fig. 5A). Analyses of cytokine expression in the liver revealed a decrease in TNFα mRNA, and significant decreases in IL-6, IL-1β and of IL-10 mRNAs in the KO mice (Fig. 5B). In contrast, the pro-inflammatory response by WBC from the KO mice remained unchanged, except IL-10 mRNA expression was significantly decreased. Most surprisingly, plasma levels for IL-6, IL-10 and TNFα were significantly higher in septic KO mice than in the WT animals, despite lower mRNA expression (Fig. 5C). Increased inflammatory markers in serum suggest a disadvantage during sepsis in the KO mice. No changes were detected for serum ALT or bacterial load in liver, spleen or blood, however (Fig. 5D). In addition, none of the KO animals died or showed signs of more severe disease than the WT mice. Hence, the
Zip14 null mutation did not appear to enhance the progression of mild sepsis within the time course used here.

Zinc supplementation reduces sepsis progression. The Zip14 KO model provided new and valuable information on the role of zinc transport to and within the liver during sepsis. To more directly characterize the effects of zinc in mild sepsis, we fed WT mice either an adequate zinc diet (ZnA) or a supplemental zinc diet (ZnH) for one week prior to CLP. Fig. 6A illustrates that zinc supplementation (ZnH) increased liver zinc in both SHAM and to a greater extent in mice by 24h after CLP. The significant drop in serum zinc following CLP was prevented in the zinc supplemented mice compared to those receiving the zinc adequate diet (ZnA). However, no significant changes for Zip14 and MT-1 mRNA expression were observed for HP, HL and WBC from mice fed the ZnA diet compared to the ZnH diet (Fig. 6B). MMP-9 mRNA was significantly decreased in HP and HL; negligible expression was detected for WBC. The ZnH diet significantly decreased IL-6 mRNA in HP, TNFα mRNA in HL and S100A9 mRNA in WBC, demonstrating the anti-inflammatory effect of zinc (Fig. 6B). Analyzing serum from ZnH mice, we found significant decreased levels of TNFα, S100A8 and S100A9 compared to ZnA mice ( 6C). Moreover, IL-6, MMP-9 and IL-10 were decreased, but not significantly. In concordance with the ameliorated inflammatory response, ALT levels in the serum were significantly decreased in the ZnH mice. Interestingly, we also found a lower bacterial load in spleen and blood when mice were fed the ZnH diet. Results clearly point to a benefit of prior zinc supplementation for the outcomes in this mouse model of sepsis.

DISCUSSION
The inflammatory acute phase response is a complex physiological process involving various cell-types and pro- as well as anti-inflammatory phases. The ultimate goal is the clearance of the insult, usually a pathogen or injury, and re-establishing homeostasis. If not re-balanced as during sepsis, tissue damage, organ failure and death are the final consequences (1, 13, 23). In this report we connect inflammation of sepsis to changes in zinc homeostasis. The murine model used here focuses primarily on sepsis up to 24h after CLP where leukocytosis occurs and where inflammatory stimuli increase including IL-6, IL-1β and TNFα. Zinc is needed for liver metabolism and protection; however the exact role this micronutrient plays in the immune response to sepsis is unknown.

Unique to this study was the identification of a cascade of Zip4, Zip6 and Zip10 zinc transporters expressed in the liver of septic mice. These changed on a temporal basis and were cell-type specific, but played only a minor role in generating hypozincemia. Zip14 was the major transporter responsible for zinc redistribution during sepsis. Therefore, this polymicrobial sepsis model is in agreement with results using LPS-induced endotoxemia (2, 30, 31) and for hepatocytes in vitro (30, 31). In addition, here we narrowed the main function in regulating zinc homeostasis to hepatocytes, while hepatic leukocytes were mostly responsible for driving inflammation expressing higher IL-1β, TNFα, S100A8 and MMP-8. While elevation of those factors during sepsis had been shown, they have not been assigned to have originated in specific cell types of the liver and not previously related to zinc redistribution.

Of significance was the inverse correlation of WBC content in the blood to the decreased serum zinc. This finding supports the hypothesis that hypozincemia is an
activating signal for the immune system (28). Not only the number but also the activity of WBC was altered during sepsis most importantly increasing expression of pro-inflammatory mediators. Associated with the acute changes in zinc distribution *in vivo* were increased mRNAs for TNFα, IL-1β, calprotectin, IL-10 and MMP-8 and -9 in WBC. The high expression of IL-6 by liver cells but not WBC suggests that this cytokine functions in an autocrine fashion to generate the rapid and strong responses observed during sepsis, which makes it a valuable target for sepsis therapy (6, 35). A similar IL-6 expression profile was observed for hepatic leukocytes. The uptake of high amounts of zinc by hepatocytes may deprive other cell populations of zinc in the systemic circulation. Our evidence in support of that idea is the increase in MT-1 mRNA and labile Zn concentration in hepatocytes compared to hepatic leukocytes (Fig. 3B). Of note were high levels of TNFα, IL-1β expression by hepatic leukocytes, indicating that restriction of zinc availability might be necessary for induction of specific cytokine expression in leukocytes. The possibility that systemic zinc deficiency may activate cytokine expression/secretion consequences has been suggested by several *in vitro* studies (21).

To approach an explanation for the significance of zinc uptake into the liver during sepsis, we analyzed Zip14 KO and WT mice. As was reported for LPS stimulation of Zip14 expression in liver (2), STAT3 phosphorylation increased after CLP. The activation may be similar in KO and WT mice which require further analysis, however. Knocking out Zip14 expression eliminated the robust increase in hepatic zinc accumulation of the WT mice following CLP. The hypozincemic response observed in the WT mice over 48h after CLP was less in the KO mice and a different response
pattern was evident. Notably, the null mutation prevented the increase in WBC for at least 24h after induction of sepsis by CLP. We interpret this to indicate that in early sepsis there is a greater metabolic flux of plasma zinc to various cells including to hepatocytes of the liver. With time in the KO model the WBC population is able to overcome that transient zinc deficit and there is an increase in WBC numbers in the systemic circulation. Further research is needed to define the mechanism.

The very robust increases of MT-1 and hepcidin in the KO mice following CLP were somewhat surprising as both are usually directly connected to major changes in zinc and iron homeostasis, respectively. In vitro and in vivo studies have shown ZIP14 can transport non-transferrin bound iron and manganese under some circumstances (4, 18, 32). Previously we demonstrated that elevated liver hepcidin expression is a characteristic of the Zip14 KO phenotype (2). In studies using the Zip14 KO model, but focused on the intestine, increased MT-1 mRNA was observed, as was extensive accumulation of labile zinc in endosomes, with modest increases in total tissue zinc content (20). The high liver MT-1 mRNA expression observed here, suggests an abnormally high level of MTF-1 activation in hepatocytes of KO mice. In that regard, in vitro evidence suggests that the hepcidin gene may be MTF-1 regulated (5).

Alternatively, since hepcidin is up-regulated by IL-6 (34), the high serum IL-6 levels in the Zip14 KO mice would yield greater than normal hepcidin expression. We propose that high hepcidin mRNA observed may reflect higher than normal circulating hepcidin and would explain why the hepatic NHI concentrations were not influenced in either the WT or KO mice with the CLP model used here. Of note, the role of hepcidin in sepsis has been viewed as generally protective (48).
Clinical evidence with humans suggest lower plasma TNFα, IL-6 and IL-10 levels correlate with survival in severely septic patients (47). Similarly, plasma TNFα correlates with severity of sepsis in human patients (12). Our data with murine sepsis shows that both hepatocytes and hepatic leukocytes contribute to systemic IL-6 levels, while hepatic leukocytes contribute to systemic TNFα, and IL-10. Survival was not an issue in our experiments since relatively mild conditions were used. Comparison of TNFα, IL-6, and IL-10 transcripts in liver cells of KO vs WT mice after CLP vs serum protein levels show opposite effects. This could relate to the times of RNA sampling vs. serum sampling. Expression by splenocytes would be an explanation as well. Alternatively, mechanisms mediating resolution of the inflammatory response including expression of antagonists to pro-inflammatory mediators including IL-1 receptor antagonist, IL-6 receptor and TNF-receptor might be disturbed. These possibilities remain to be tested. Nevertheless, the marked increases in serum TNFα, IL-6, and IL-10 suggest that progression of sepsis is influenced by Zip14 expression. More extensive studies are needed to evaluate the physiologic role of ZIP14 in a severe sepsis model. In addition, in future studies the influence of gender needs to receive attention using this mutant model. Nevertheless, our data support the concept that zinc, as transported by ZIP14, influences cytokine levels through as yet unidentified mechanism during sepsis. Our finding that supplemental dietary zinc generally reduces cytokine levels in CLP mice supports this hypothesis.

Evidence from genome expression assays indicate that metallothionein mRNAs and Zip8 mRNA were among highly up-regulated genes in non-survivors of pediatric septic shock (46). Those patients exhibited hypozincemia, while surviving patients had
normal serum zinc levels. Those findings support the idea that a zinc-related component is involved in sepsis severity. At first glance accumulating high amounts of zinc might be surprising, as it could be the perfect environment also for bacterial growth. However, our data revealed that most of the zinc is directly taken up by the hepatocytes, possibly causing a transient zinc deficiency in the intracellular space and blood vessels supplying the liver. The highly compromised transfer of zinc to the liver in the KO mice did not lead to cellular damage, based on serum ALT levels. However, greater PD-L1 expression supports the notion that the hepatocytes are in a protective metabolic state (49).

Dietary zinc deficiency has been shown to accentuate organ damage in a murine model of severe sepsis resulting in high mortality (25). Conversely, short term dietary zinc supplementation (three days) in another model of severe murine sepsis decreased mortality and lowered indices of sepsis including bacterial load (36). The mechanism of the influence of zinc on sepsis is not fully known. The matrix metalloproteases play a role in tissue injury of sepsis (33, 42). Calprotectin (S100A8/9) inhibits microbial growth through zinc chelation (9). Calprotectin may also inhibit MMPs by binding zinc that is essential for enzymatic activity (24). Zinc may also inhibit cytokine production through a variety of mechanisms (16, 21). Our very positive effects of zinc supplementation suggest that timing of zinc as a therapy is very important. In a clinical setting with human septic patients zinc supplementation could be monitored by some approach such as measurement of PBMC metallothionein mRNA levels (2).

There has been some controversy regarding the value of murine models of human sepsis (39, 41). Those studies were conducted with PBMCs as a source of the
RNA used for transcriptome profiling by microarrays. Nevertheless, significant similarities exist as mRNAs for MMPs, calprotectins, specific cytokines and nutrient transport proteins in both mice and human PBMCs were detected by those profiling experiments (41). Consequently, significant advances continue to be made with the murine CLP-induced sepsis model, for example, the role played by IL-3 (44) and TNFR shedding from hepatocytes (12). This is particularly the situation when experiments are at the organ/tissue level, e.g. the liver, as shown in our present experiments, rather than that of transcriptome analysis using circulating blood cells.

The focus of this report is on the liver which is considered as a key organ to undergo dysfunction in sepsis (6, 35). The experiments presented here, the first with a zinc transporter knockout model, suggest that zinc and the zinc transporter ZIP14 influence aspects of the pathophysiology of nonlethal polymicrobial murine sepsis induced by CLP. Ablation of Zip14 produced a delay in development of leukocytosis, prevented liver zinc accumulation, altered the kinetics of hypozincemia and drastically increased serum IL-6, TNFα, and IL-10 concentration following CLP. To identify zinc-responsive factors modulated by acute sepsis mice were fed zinc supplemented diets for one week. The supplemental zinc reduced cytokines, calprotectins (S1008/9), serum ALT and blood bacterial loads. Supplemental zinc at this level did not appear to produce toxicity. These data indicate zinc exercises an anti-inflammatory role during sepsis by attenuating the proinflammatory response and that ZIP14, when located at the cell surface, produces an important transport function to get zinc into cells for sites of action.

ACKNOWLEDGEMENTS
We acknowledge the scientific contributions and advice of Drs. Tolunay B. Aydemir, Catalina Troche, and Shou-Mei Chang throughout this project and Laura Orta for manuscript preparation. Present address of Dr. I. Wessels is Institute of Immunology, Aachen University Hospital, Aachen, Germany.

GRANTS

The research was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant (RO1DK94244) and the Boston Family Endowment Funds of the University of Florida to R.J. Cousins. Dr. Inga Wessels was supported by a Deutsche Forschungsgemeinschaft Fellowship (WE 5329/1-1).

DISCLOSURES

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

IW and RJC planned the research, IW conducted the research and IW and RJC wrote the manuscript. Both authors approved the final manuscript.

Figure Legends

Fig. 1. CLP-induced sepsis alters zinc distribution gene expression patterns in liver tissue and serum sepsis markers. Mice were killed at 1h, 3h, 9h, 24h, 48h and 72h after SHAM or CLP surgery. A: Time course of zinc concentrations in whole liver and serum, white blood cell counts, liver MT-1 mRNA expression, and WBC MT-1 mRNA and labile intracellular zinc levels. CLP data were normalized to SHAM-operated mice. Values are means +/- SEM (n = 4 - 5 mice per group). B: IL-6, TNFα, IL-10, MMP-9, S100A8 and S100A9 mRNA expression in whole liver. Values were normalized over TBP as housekeeping gene. Values are means +/- SEM (n = 3–12 mice per group). C:
Cytokine concentrations for IL-6, MMP-9 and S100A8 in the serum were analyzed using a custom Luminex Assay. Values are means +/- SEM (n = 3 – 7). Significant differences were determined by ANOVA/ Bonferroni test. *P < .05, **P < .01 and ***P < .001 different from the SHAM-operated mice.

Fig. 2. CLP – induced elevation of liver zinc is correlated with elevated Zip14 expression. Mice were killed at 1h, 3h, 9h, 24h, 48h and 72h after SHAM or CLP operation. A: Whole liver Zip4, Zip6, Zip8, Zip10, and Zip14 mRNA expression were analyzed and normalized to TBP mRNA expression. Values are mean quotients of CLP/SHAM +/- SEM (n = 3–12 mice per group). Significant differences were determined using ANOVA/Bonferroni test *P < .05 and **P < .01. B: Western analysis of Zip14 expression in the liver of CLP and SHAM operated mice 24h, 48h and 72h after surgery. The boxes around the CLP and Sham blots at the 24h time point indicate the noncontiguous lanes were derived from the same gel. Shown are representative blots of multiple experiments.

Fig. 3. Functions influencing hepatic zinc metabolism and the hepatic immune response are cell-specific. Mice were killed 1h, 3h and 9h after CLP and hepatocytes (HP) and hepatic leukocytes (HL) were separated by Percoll gradient centrifugation. A: Hepcidin is solely expressed in hepatocytes whereas hepatic leukocytes majorly express F4/80. Values were normalized over TBP B: MT-1 and Zip14 mRNAs. Labile intracellular zinc concentrations were assessed via flow cytometry using FluoZin3. C: Zip4, Zip6 and Zip10 mRNA. D: IL-6, TNFα, IL-1β, S100A8, IL-10, MMP-8 mRNAs. Values are means +/- SEM (n =3 – 7 mice per group). *P < .05 and **P < .01 different from the hepatocytes (HP) for each time point (ANOVA/Bonferroni test).
Fig. 4. Zinc transfer to the liver in Zip14 KO mice and altered zinc homeostasis

Zip14−/− KO and WT mice were killed 9h, 24h, 48h, and 72h after CLP or SHAM operation. A: Serum zinc concentrations and WBC. Zip4, Zip6, Zip8, Zip10 and ZnT1 mRNA expression in liver. mRNA values were normalized to TBP and the SHAM operated animals. Finally, the quotient for KO/WT was calculated. B: Liver zinc and MT-1 mRNA. C: Liver hepcidin mRNA and NHI. D: PD-L1 and PCNA mRNA expression in liver and WBC. Values are means +/- SEM (n = 3 mice per group). *P < .05, **P < .01, and ***P < .001 different from the KO animals (ANOVA/Bonferroni test).

Fig. 5. Zip14 ablation alters the hepatic inflammatory response to sepsis.

Zip14−/− KO mice and WT mice were killed 24h after CLP or SHAM operation. A: Western analysis of ZIP14 expression and STAT3 phosphorylation in the liver of CLP compared to SHAM operated mice. Shown are representative examples of n = 3 independent experiments using tubulin and STAT3 as loading controls. B: IL-6, TNFα, IL-1β and IL-10 mRNA expression in liver and WBC. CLP values were normalized over TBP. Values are means +/- SEM (n = 3 mice per group). C: Cytokine concentrations for IL-6, MMP-9, S100A8 and S100A9 in the serum were analyzed using a custom Luminex Assay. TNFα and IL-10 plasma concentrations were analyzed by ELISA. Results are shown as means +/- SEM (n = 3 mice per group). D: Serum ALT activity. Values are shown as means +/- SEM (n = 3 -4). Values that are significantly different do not share the same letters. Bacterial load of liver homogenate, spleen homogenate and whole blood is shown. Results are shown as means +/- SEM (n = 3 mice per group). Statistical analysis was with ANOVA/Bonferroni (B,C) OR ANOVA/ Tukey’s (D). *<.05 different from KO mice.
Fig. 6: Zinc-supplementation limits hyperinflammation and positively influences the anti-microbial response. Mice were fed with zinc adequate (ZnA) vs zinc high (ZnH) diet for 7 days and were killed 24 h after subject to CLP or SHAM operation. A: Liver and serum zinc concentrations of the mice B: Zip14, MT-1, MMP-9, IL-6, TNFα and S100A9 mRNA expression in hepatocytes (HP), hepatic leukocytes (HL) and WBC. Values were normalized over TBP as housekeeping gene. C: IL-6, MMP-9, S100A8 and S100A9 concentrations in the plasma were analyzed using a custom Luminex Assay. TNFα and IL-10 plasma concentrations were analyzed by ELISA. D: Serum alanine aminotransferase (ALT) and bacterial load of liver homogenate, spleen homogenate and whole blood were measured. Results are shown as means +/- SEM (n = 3-5 mice per group). *P<.05 and **P<.01 different from the SHAM-operated mice (ANOVA/Bonferroni, was used).

REFERENCES


Figure 2

CLP | SHAM
---|---

<table>
<thead>
<tr>
<th>24h</th>
<th>ZIP14</th>
<th>Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>48h</th>
<th>ZIP14</th>
<th>Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>72h</th>
<th>ZIP14</th>
<th>Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>
Figure 4
Figure 5

A

<table>
<thead>
<tr>
<th>wt</th>
<th>KO</th>
<th>wt</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zip14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>mRNA [CLP/SHAM]</th>
<th>IL-6</th>
<th>TNFα</th>
<th>IL-1β</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>KO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>mRNA [CLP/SHAM]</th>
<th>IL-6</th>
<th>TNFα</th>
<th>IL-1β</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>KO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Serum Protein [CLP/SHAM]</th>
<th>IL-6</th>
<th>S100A8</th>
<th>S100A9</th>
<th>MMP9</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>KO</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein [CLP/SHAM]</th>
<th>TNFα</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>KO</td>
<td></td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>Serum ALT [IU/L]</th>
<th>SHAM</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>KO</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bact. Load [log of cfu/mL]</th>
<th>Liver</th>
<th>Spleen</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO CLP</td>
<td>WT CLP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6

A

Liver Zinc (mmol/L)

B

Zn14g/TBP

C

IL-6/TBP

D

IL-10 (ng/mL)

E

Serum ALT (IU/L)

Bacterial load (CFU/mL kg 10)

TnF-α (pg/mL)

WBC

HP

IL-1β (pg/mL)

HL

IL-10 (ng/mL)

WBC

NBP-9 (TBP)

** P<0.01

a P<0.05

b P<0.001

c P<0.0001

D

Entire study

E

Liver

Serum

Blood

CLP

SHAM