Zinc modulates the innate immune response in vivo to polymicrobial sepsis through regulation of NF-κB

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Bao S, Liu M-J, Lee B, Besecker B, Lai J-P, Guttridge DC, Knoell DL. Zinc modulates the innate immune response in vivo to polymicrobial sepsis through regulation of NF-κB. Am J Physiol Lung Cell Mol Physiol 298: L744–L754, 2010. First published March 5, 2010; doi:10.1152/ajplung.00368.2009.—Zinc is an essential element that facilitates coordination of immune activation during the host response to infection. We recently reported that zinc deficiency increases systemic inflammation, vital organ damage, and mortality in a small animal model of sepsis. To investigate potential mechanisms that cause these phenomena, we used the same animal model and observed that zinc deficiency increases bacterial burden and enhances NF-κB activity in vital organs including the lung. We conducted further studies in the lung to determine the overall impact of zinc deficiency. At the molecular level, NF-κB p65 DNA-binding activity was enhanced by zinc deficiency in response to polymicrobial sepsis. Furthermore, expression of the NF-κB-targeted genes IL-1β, TNFα, ICAM-1, and the acute phase response gene SAA1/2 were elevated by zinc deficiency. Unexpectedly, the amount of NF-κB p65 mRNA and protein was increased in the lung including alveolar epithelia of zinc-deficient mice. These events occurred with a significant and concomitant increase in caspase-3 activity within 24 h of sepsis onset in zinc-deficient mice relative to control group. Short-term zinc supplementation reversed these effects. Reconstitution of zinc deficiency in lung epithelial cultures resulted in similar findings in response to TNFα. Taken together, zinc deficiency systemically enhances the spread of infection and NF-κB activation in vivo in response to polymicrobial sepsis, leading to enhanced inflammation, lung injury, and, as reported previously, mortality. Zinc supplementation immediately before initiation of sepsis reversed these effects thereby supporting the plausibility of future studies that explore zinc supplementation strategies to prevent sepsis-mediated morbidity and mortality.

NF-κB pathway; lung; inflammation

SEPSIS IS A FREQUENTLY OCCURRING SERIOUS MEDICAL CONDITION CAUSED BY INFECTION LEADING TO SYSTEMIC ACTIVATION OF THE HOST INFLAMMATORY RESPONSE AND TISSUE INJURY. SUBSEQUENT FAILURE OF VITAL ORGANS, INCLUDING THE LUNG, IS THE LEADING CAUSE OF MORTALITY. To begin to understand the potential impact of zinc deficiency on sepsis, we studied a small animal model of sepsis. To investigate potential mechanisms that cause these phenomena, we used the same animal model and observed that zinc deficiency increases bacterial burden and enhances NF-κB activity in vital organs including the lung. We conducted further studies in the lung to determine the overall impact of zinc deficiency. At the molecular level, NF-κB p65 DNA-binding activity was enhanced by zinc deficiency in response to polymicrobial sepsis. Furthermore, expression of the NF-κB-targeted genes IL-1β, TNFα, ICAM-1, and the acute phase response gene SAA1/2 were elevated by zinc deficiency. Unexpectedly, the amount of NF-κB p65 mRNA and protein was increased in the lung including alveolar epithelia of zinc-deficient mice. These events occurred with a significant and concomitant increase in caspase-3 activity within 24 h of sepsis onset in zinc-deficient mice relative to control group. Short-term zinc supplementation reversed these effects. Reconstitution of zinc deficiency in lung epithelial cultures resulted in similar findings in response to TNFα. Taken together, zinc deficiency systemically enhances the spread of infection and NF-κB activation in vivo in response to polymicrobial sepsis, leading to enhanced inflammation, lung injury, and, as reported previously, mortality. Zinc supplementation immediately before initiation of sepsis reversed these effects thereby supporting the plausibility of future studies that explore zinc supplementation strategies to prevent sepsis-mediated morbidity and mortality.

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tion of bacterial clearance and the proinflammatory response, culminating in increased tissue injury. Our focus in this investigation was placed on the lung knowing that it is typically the first vital organ adversely affected by overwhelming inflammation during the early stages of sepsis.

MATERIALS AND METHODS

Animal Studies

Establishing a mouse model of zinc deficiency. Ten-week-old adult male C57BL/6 mice (~25 g; Harlan Sprague-Dawley, Indianapolis, IN) with fully developed lungs were randomly placed on a zinc-deficient diet (1 part per million (ppm); TD.85419; Harlan Teklad) or a matched control diet (50 ppm; TD.85420) for 3 wk, a time sufficient to establish subacute zinc deficiency without requiring pair feeding. A zinc-free environment was carefully maintained using dezoinized water in zinc-free containers and stainless steel cages along with daily cage changes. Plasma zinc concentrations and tissue metallothionein levels were evaluated in the lung and liver to confirm that animals achieved a zinc-deficient state (27). As previously reported, zinc-deficient diets consistently resulted in an ~2.5-fold decrease in plasma zinc levels as measured by atomic absorption spectroscopy. An additional group received a zinc-fortified diet (100 ppm; TD.07129) for 3 days following an 18-day zinc-deficient regimen (27). Plasma zinc levels consistently returned to normal by the end of the 3-day supplementation regime. Importantly, we reassessed zinc levels in an identical fashion in the repeated studies and recorded similar results (data not shown).

CLP. At the end of the dietary regime, mice were subjected to CLP via laparotomy under general anesthesia (1% isoflurane) as described previously (10, 23) with slight modifications. Briefly, through an upper midline abdominal incision, the cecum was delivered, ligated with a silk suture 1 cm from the tip, and doubly punctured with a 21-gauge needle. After puncture, the cecum was gently squeezed to extrude fecal content and returned to the abdominal cavity. The laparotomy was then closed. This model reproducibly results in ~30% mortality within 7 days in our hands. It is important to note that we previously reported that sham surgery (delivery of the cecum without ligation and puncture) had no effect on indices of inflammation, vital organ injury, and mortality so was not further evaluated in this investigation. Animal studies were conducted in accordance with prior approval by The Ohio State University Institutional Animal Care and Use Committee. Blood, lung, liver, spleen, and intestine were collected at 6 or 24 h after CLP. Lung tissue was processed as follows. Immediately after death, the thoracic cavity was opened, and the lung vasculature was lavaged with saline. The left ventricle was cut open to allow perfusion through the right ventricle with 30 ml of PBS. One half of the lung was excised and submitted to RNA or protein extraction. The other half was prepared for fixation by inflation with PBS-buffered formaldehyde at a constant pressure equal to 25 cmH2O, which allows the homogenous expansion of the lung parenchyma. Lung tissue was then embedded in paraffin.

Bacterial Colony Counts

Blood was collected at 24 h after CLP and immediately plated on blood agar plates (TSA II, Trypticase Soy Agar with 10% Sheep Blood; Becton Dickinson) in 5 serial 10-fold dilutions. Following overnight incubation, bacterial colonies were enumerated and compared among different treatment groups.

Imaging and Luciferase Measurements

Transgenic mice (Caliper Life Sciences, Hopkinton, MA) that systemically express luciferase under the control of an NF-κB-responsive promoter were used (8) to determine real-time in vivo imaging of NF-κB activity in intact animals and vital organs. Imaging of transgenic mice was performed with an ultrasensitive camera consisting of an image intensifier coupled to a charge-coupled device (CCD; Xenogen IVIS Imaging System; Xenogen, Alameda, CA). Transgenic mice were administrated different zinc diets and then subjected to CLP as previously described. Whole body imaging was conducted at 4 and 8 h post-CLP. Before imaging, mice were anesthetized under isoflurane. D-luciferin (100 mg/kg; Caliper Life Sciences) dissolved in 200 μl PBS, pH 7.8, was injected intraperitoneally. Grayscale images were obtained for reference. Luminescence emitted from each animal was integrated for 10 min starting 2 min after D-luciferin injection. Following that, individual vital organs were excised from the mice and then placed on six-well plates and immediately imaged. The pseudocolored images represent light intensity. All images were processed with Igor Pro 4.09A and Xenogen Living Image 2.50 software programs (Xenogen).

Evaluation of NF-κB DNA-Binding Activity

The DNA-binding activity of NF-κB in mouse lung tissue lysate was quantified by the TransAM NF-κB p65 transcription factor assay kit using an ELISA-based format (Active Motif North America, Carlsbad, CA). Mouse lung extracts were incubated in 96-well plates coated with the immobilized oligonucleotide (5'-AGTGGAGG-GACTTTCCAGGC-3') containing a consensus (5'-GGGACTT-TCC-3') binding site for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary antibody (Ab) specific for the activated form of p65, visualized by anti-IgG horseradish peroxidase conjugate and developing solution, and quantified at 450 nm with a reference wavelength of 655 nm. The background binding, obtained by incubation with a two-nucleotide mutant oligonucleotide (5'-AGTGTAGGCCACTTTCCAGGC-3') was subtracted from the value for binding to the consensus DNA sequence.

Real-Time RT-PCR

Total RNA was isolated from lung tissue or cell culture using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed by using ThermoScript RT-PCR System (Invitrogen). Real-time PCR was performed with the 7900HT Fast Real-Time PCR system (Applied Biosystems) using SYBR Green reagents. All the analysis involving mouse lung tissue was normalized against the average cycle threshold number of mouse GAPDH and cyclophilin genes. Studies involving human lung epithelial cell culture were normalized against a human GAPDH probe. The sequences of all the PCR primers are available on request.

Immunohistochemistry and Immunofluorescent Staining of Mouse Lung Tissue

Lung tissue slides were deparaffinized and rehydrated, and then antigen retrieval was performed. Following permeabilization with 1% Triton X-100 and blocking with 2% goat serum in PBS, the sections were incubated with primary rabbit anti-p65 Ab (1:200; Rockland, Gilbertsville, PA; provided by Dr. D. Gutridge, The Ohio State University), which detects total NF-κB p65 protein, and then incubated with the secondary Ab, followed by counterstaining with hematoxylin and microscopic evaluation. For immunofluorescence staining, sections of tissues or cells were incubated with the primary rabbit anti-p65 Ab followed by the secondary Ab (Alexa Fluor 488 goat anti-rabbit Ab; Invitrogen). Nuclear DNA was detected with 4′,6′-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). Rhodamine-labeled Ricinus communis agglutinin I (RCA), a marker for type I lung epithelial cells, was also used for colocalization imaging studies. Before inspection, slides were mounted with Citifluor antifade mounting medium (AF1; Electron Microscopy Science) and then examined using a disk scanning confocal microscope (Olympus BX61). p65 Protein levels in the lung were quantified using histogram analysis in Adobe Photoshop CS2 (Adobe Systems). Brown
pixels, representing positive staining for NF-κB p65 protein, were enumerated.

**Cell Culture, Transfection, and Luciferase Assay**

The human lung epithelial cell lines A549 and BEAS-2B [American Type Culture Collection (ATCC), Manassas, VA] were routinely maintained in DMEM supplemented with 10% FBS, 1% sodium pyruvate, 0.1 mg/ml streptomycin, and 100 IU/ml penicillin at 37°C in a 5% CO₂-humidified atmosphere. Primary human lung epithelial cells (hLECs) were isolated after enzymatic dissociation from trachea, bronchi, and bronchioles of adult donor lungs, seeded onto collagen-coated, semipermeable membranes (0.6 cm²; Millicell HA; Millipore, Bedford, MA), and grown as fully differentiated, polarized, monolayer cells at an air-liquid interface, as previously described (4, 25). Transient transfection was performed with Lipofectamine 2000 (Invitrogen) in A549 and BEAS-2B cells seeded onto 48-well plates. For each well, the plasmid-Lipofectamine complexes were formed by incubating 0.3 μg of NF-κB 3×κB-luc reporter plasmid [provided by Dr. D. Guttridge, The Ohio State University, as previously described (16)], 0.1 μg of promoter-linked Renilla luciferase vector (pRL-TK) plasmid, and 1 μl of Lipofectamine at room temperature for 20 min in a total volume of 100 μl of serum-free DMEM. The DNA-Lipofectamine complexes were then diluted with 100 μl of DMEM and applied at 200 μl per well. After 12 h of incubation, the transfection medium was replaced with DMEM containing 10% FBS with the appropriate solvent or reagents. Cell lysis was performed 6 h after transfection and then subjected to a luciferase reporter gene assay using the Dual-Glo Luciferase Assay System (Promega, Madison, WI).

**Cytokine Analysis**

Multiplex quantification of cytokine and chemokine levels was conducted in hLEC supernatants obtained 24 h after exposure to either TNFα alone (10 ng/ml) or in combination with the zinc chelating agent N,N,N′,N′-tetraakis(2-pyridylmethyl)ethylenediamine (TPEN; 20 μM) or zinc sulfate (5 μM). The concentration of each cytokine was analyzed from a 50-μl sample volume using a custom-made Bio-Plex Cytokine singleplex panel that includes IL-6 and ICAM-1 using the Bio-Plex 200 Analysis System (Bio-Rad, Hercules, CA).

**SAA Determination**

Approximately 0.5 ml of whole blood was collected via cardiac puncture using a 25-gauge × 5/8 in. needle. Serum was separated by centrifugation at 1,000 g for 10 min at 4°C. Quantification of serum amyloid A (SAA) levels was determined by ELISA according to the manufacturer’s recommendations (Invitrogen BioSource).

**Measurement of Caspase-3 Activity**

Caspase activity was measured with 7-amino-4-trifluoromethyl coumarin (AFC). For all AFC preparations, the presence of active caspase-3 in lung cell lysates was determined by an AFC assay using specific fluorosubstrates. Lysates were incubated with cyto-buffer (1 mM DTT and 20 mM PIPES, pH 7.0, and 1 mM EDTA) containing 1 mM DTT and 20 μM Asp-Glu-Val-Asp (DEVD)-AFC or Ile-Glu(OMe)-Thr-Asp(OMe)-AFC (Enzyme Systems Products). The release of free AFC was determined using a fluorometer (400-nm excitation and 505-nm emission; Cytofluor 4000; Perseptive, Framingham, MA).

**Statistical Analysis**

All data are presented as means ± SD. Statistical comparisons among different groups were performed using ANOVA. Systemic bacterial burden between all groups was evaluated by ANOVA followed by Tukey honestly significant difference test. Significance was assumed at a P value of <0.05.

**RESULTS**

**Zinc Deficiency Increases Bacterial Burden and NF-κB Activity in Response to Sepsis In Vivo**

As previously described, mice were randomized into 3 groups and received a normal diet, a zinc-deficient diet, or a zinc-deficient diet followed by a short repletion period. At 24 h post-CLP, mice were killed; blood samples were obtained, plated on blood agar plates, and then enumerated for bacterial colony counts the following day. As shown in Fig. 1, zinc-deficient (Zn⁻/CLP) mice consistently exhibited higher bacterial counts within their bloodstream compared with zinc-sufficient (Ctrl/CLP) mice. Short-term zinc supplementation (Zn⁺/CLP) lowered the bacterial counts compared with the Zn⁻/CLP group. No bacteria were found in animals placed only on dietary modification without CLP (data not shown). Although
Fig. 2. Zinc deficiency increases NF-κB activation systemically following CLP. A: a representative whole body image obtained from 1 mouse within each treatment group is shown and includes the untreated control diet (Ctrl) group along with analysis of mice 8 h after CLP in conjunction with 3 different zinc diets, including the control diet (Ctrl/CLP), a zinc-deficient diet (Zn−/CLP), or a zinc-deficient diet followed by acute zinc supplementation (Zn+/CLP) (n = 2 for untreated mice, n = 3 per dietary groups involving CLP treatment). The anesthetized mice were placed in a light-sealed chamber connected to the charge-coupled device (CCD) camera for image analysis. Luminescence emitted from each animal was integrated for 10 min starting 2 min after D-luciferin injection. B: vital organs including the liver, lung, spleen, and intestine were excised from each animal and immediately subjected to quantitative bioluminescent imaging. The composite images of each tissue from each animal are presented after color scale adjustment (the scale for A and B is identical). C: metric analysis of total photon flux of pooled data for each tissue from each treatment group (*P < 0.05, #not statistically significant).
the comparison between Zn−/CLP and Ctrl/CLP mice did not achieve statistical significance due to interindividual variability within treatment groups, the bacterial counts in the Zn−/CLP mice were all substantially elevated. Next, BALB/c NF-κB luciferase transgenic mice were employed to monitor NF-κB activation in vivo during the early stages of CLP-induced polymicrobial sepsis. Briefly, a total of 11 transgenic mice were randomized into 3 groups and received a normal diet (n = 5), a zinc-deficient diet (n = 3), or a zinc-deficient diet followed by a short repletion period (n = 3) identical to that described for use with C57BL/6 mice (MATERIALS AND METHODS). Following the dietary period, a combined total of 9 mice were subjected to receive CLP. At 8 h after CLP treatment, the mice received an intraperitoneal injection of luciferin and then were placed posteriorly in a light-sealed chamber and imaged. Initially, we observed a low level of luminescence restricted primarily within the neck region of noninfected control mice consistent with a previous report by an independent group (8). In direct comparison, a very intense signal was detected within the abdominal region in the CLP-treated mice. In addition, luminescence was also observed within the thoracic region. The luminescence in the neck was associated with lymph nodes, the thoracic signal originated from thymus and lung, and the abdominal signal coming from the intestine, presumably originating from the site of CLP. The Zn−/CLP mice consistently had increased bioluminescence in the abdominal and thoracic cavity compared with the other treatment groups as shown by representative images (Fig. 2A). To directly quantify the signal within vital organs, the lung, liver, spleen, and intestine were excised immediately following whole body imaging, and luminescence was measured (Fig. 2B). Quantitative analysis of each vital organ from each animal consistently demonstrated that Zn−/CLP mice had a significant increase in NF-κB-dependent luminescence across all organs studied compared with Ctrl/CLP mice (Fig. 2C). Furthermore, we observed that short-term oral zinc repletion (Zn+/CLP) reduced NF-κB-dependent luminescence in all vital organs studied. Taken together, these findings demonstrate that zinc has a systemic impact on the NF-κB-mediated innate immune response during the early stages of polymicrobial infection.

Zinc Deficiency Increases NF-κB Activity in the Lung

Since the lung is the first vital organ adversely affected at the onset of sepsis, and the extent of acute lung injury directly correlates with mortality (1), we next determined whether zinc deficiency causes more extensive inflammation and injury in the lung through enhancement of NF-κB activity. To test this, we first determined whether lung homogenates obtained from C57BL/6 adult mice (randomized and treated as mentioned in MATERIALS AND METHODS) possessed different levels of active NF-κB p65 using the TransAM DNA-binding assay. Lung tissue lysates were obtained from mice on normal and modified zinc diets at 6 and 24 h after CLP treatment. Zn−/CLP mice exhibited increased NF-κB p65 DNA-binding activity in the lung (Fig. 3). Consistent with our previous observation, a significant increase in NF-κB activation was apparent very early (at 6 h) in the lung, suggesting that zinc deficiency likely accelerates the time course of sepsis-initiated NF-κB activation in key vital organs distal to the original site of infection (cecum). Zinc supplementation suppressed the extent of NF-κB activation in the lung at all time points studied.

NF-κB initiates the host innate immune response (40) and, in doing so, activates the expression of many genes including but not limited to cytokines, chemokines, acute phase response proteins, as well as NF-κB family members. Based on this, we observed that the expression of NF-κB-regulated genes was increased in the lung of septic mice within 24 h (Fig. 4). The transcript levels of cytokines including IL-1β and TNFα, factors that further enhance NF-κB activation via a positive feedback loop, were increased in Zn−/CLP mice. A similar effect was observed with NF-κB/Rel family members including p50 (NFκB1) and IκB family members including IκBα (NFκBIA) (14, 20, 41). In addition, we observed an increase in the expression of the chemokines CXCL14 and CCL3 as well as ICAM-1 (CD54), all of which are involved in leukocyte recruitment during inflammation (34). The expression of all the evaluated genes was enhanced in response to zinc deficiency (Zn+/CLP) and decreased by zinc supplementation (Zn−/CLP). This is also consistent with the results of p65 DNA-binding activity, thereby demonstrating that in the context of CLP-induced sepsis, zinc deficiency enhances immune activation in the lung.

The NF-κB pathway is also centrally involved in activation of the acute phase response following infection (12, 29). SAA1/2 is the most highly expressed acute phase response protein and has recently been reported to play an integral role in pathogen recognition and immune activation (19), so we determined whether SAA expression was modulated in the lung by zinc nutritional status in response to sepsis. As shown, SAA1/2 gene expression was significantly induced in the lung by zinc deficiency compared with Ctrl/CLP mice (Fig. 5A). Consistent with this finding, circulating plasma SAA1/2 levels were also increased (Fig. 5B). Collectively, these data demonstrate that zinc status also modulates the acute phase response to sepsis by increasing SAA1/2 expression, a known target gene of NF-κB (29).
Zinc Deficiency Increases NF-κB p65 in Lung Parenchyma

Consistent with mRNA data, immunostaining revealed that the Zn⁻/CLP treatment group had a significant increase in NF-κB p65 total protein, which was dispersed throughout the entire lung compared with other groups (Fig. 6A). Quantitative analysis of lung tissue confirmed a statistically significant increase in p65 protein (Fig. 6B). To further determine the localization of p65 expression in the lung, immunofluorescent confocal microscopy of similar specimens was conducted. As shown, p65 (green) was most abundant in zinc-deficient animals following CLP and was localized in both nuclear and cytoplasmic compartments of lung cells (Fig. 6C). A significant portion of p65-positive cells were alveolar lung epithelia, as determined by colocalization of p65 in cells staining positive for RCA (red), an alveolar epithelial type I cell-specific marker. We also observed that a portion of positive cells were nonparenchymal and presumably macrophages based on morphological appearance and anatomic location. These results provide evidence that NF-κB p65 expression is significantly increased within the lung as a consequence of zinc deficiency and thereby amplifies innate immune activation during the early stages of sepsis.

Differential Effect of Zinc on NF-κB Activity in Human Lung Epithelia

Having observed that zinc nutritional status modulates NF-κB expression and activity in the lung in vivo, we determined whether zinc modulates NF-κB activity in two human lung epithelial cell lines. As expected, we observed that TNFα triggered the rapid mobilization of endogenous NF-κB (p65) into the nucleus in both A549 and BEAS-2B cells (green fluorescence) within 15 min (Fig. 7A). In a similar manner, TNFα treatment induced NF-κB-dependent luciferase expression (3xκB-luc) in both cell lines (Fig. 7B and C). Consistent with in vivo observations and the work of others (26, 43), prior

Fig. 4. Zinc deficiency modulates NF-κB-mediated related gene expression in the lung following sepsis. C57BL/6 black mice were subjected to modified diets for 3 wk and then subjected to CLP. Mouse lungs were lavaged and perfused with saline at 24 h following CLP. Total RNA was isolated, and the expression of NF-κB-related genes was determined by real-time PCR. The genes included: the cytokines IL-1β and TNFα (A); NF-κB family p65, p50, IκBα, and IκB family member B cell lymphoma 3 (Bcl-3) (B); and chemokines CCL3 and CXCL14 and the cell adhesion molecule ICAM-1 (C). n = 5 lungs per treatment group; *Zn⁻/CLP vs. Ctrl/CLP, P < 0.05; ‡Zn⁺/CLP vs. Zn⁻/CLP, P < 0.05.

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Fig. 5. Zinc deficiency enhances the acute phase response in response to sepsis. A: the mRNA levels of serum amyloid A 1/2 (SAA1/2) were measured by real-time PCR (n = 5 animals per treatment group). B: blood was obtained from the same animals at 6 or 24 h following CLP, and SAA levels were measured by ELISA. n = 5 per group; *Zn⁻/CLP vs. Ctrl/CLP, P < 0.05; ‡Zn⁺/CLP vs. Zn⁻/CLP, P < 0.05.
treatment with the zinc-specific chelator TPEN (31) enhanced NF-κB-mediated luciferase expression in both cell lines. The addition of zinc alone or in combination with the ionophore pyrithione inhibited NF-κB-driven luciferase expression.

Confirmatory experiments were performed using primary hLECs. Chelation of intracellular zinc with TPEN before TNFα stimulation enhanced the expression of NF-κB regulatory genes including IL-6, TNFα, manganese superoxide...
dismutase (MnSOD), IL-1β, and ICAM-1 compared with cultures treated with TNFα alone (Fig. 8). We also observed a similar increase in IL-6 and ICAM-1 protein levels in supernatants. In contrast, zinc supplementation following TPEN treatment suppressed the expression of the same NF-κB-driven genes and corresponding proteins. These findings support our previous observations and demonstrate that zinc deficiency enhances NF-κB activity in human lung epithelia, in response to a cytokine that appears shortly after the onset of infection, and that zinc supplementation downregulates the immune response.

Zinc Deficiency Increases Caspase Activity

Previously, we (27) reported a marked increase in lung injury and the presence of TdT-mediated dUTP nick end labeling (TUNEL)-positive cells, consistent with vital organ dysfunction in Zn^-/-CLP animals. Based on this, the activity of caspase-3, an enzyme responsible for the execution phase of apoptosis, was
ZINC DEFICIENCY ENHANCES NF-κB ACTIVITY IN SEPSIS

In this investigation, we observed that zinc deficiency increases bacterial burden and enhances NF-κB activity in vivo thereby causing exaggeration of both the innate immune and acute phase response. Short-term zinc repletion before the onset of sepsis, with an oral regimen previously shown to normalize plasma zinc levels (27), significantly reduced the inflammatory response. This is the first report to demonstrate the effects of zinc deficiency on the innate host immune response in relation to the NF-κB pathway in a model of polymicrobial sepsis. In addition, evidence is provided to suggest that the adverse consequences of zinc deficiency are contributed to by dysregulation of the immune response by parenchymal cells within the lung. This is consistent with previous reports demonstrating that zinc deficiency increased shock and liver injury following endotoxin administration (38) and that zinc supplementation abrogates NF-κB activation in an endotoxin-induced inflammation model in mice, thereby reducing TNFα production and subsequent liver injury (50).

Zinc is an essential dietary nutrient that mediates many physiological processes. Knowing that moderate zinc deficiency is highly prevalent in populations that are also susceptible to sepsis, we pursued studies in a conventional animal model of sepsis in which we could establish a clinically relevant degree of zinc deficiency (27). This CLP model mimics many of the clinical features of sepsis in humans. The animals develop progressive polymicrobial bacteremia, cytokine, and chemokine induction, fever, and a hypermetabolic state (23). Consistent with previous studies, we observed that zinc deficiency resulted in a decrease in bacterial clearance within the first 24 h of sepsis onset (24). As shown by real-time in vivo imaging, the septic mice initiated an inflammatory response in the abdominal region shortly following surgery that rapidly disseminated to the thoracic cavity (Fig. 2A). Related to this, IL-1β and TNFα transcript levels in the lung were elevated as early as 2 h following CLP treatment showing that immune activation rapidly spreads to vital organs. When the Zn-/CLP treatment group was compared with Ctrl/CLP animals, the magnitude and rate of NF-κB activation were both significantly increased. The function of zinc as an immunomodulator is controversial since it has been identified as both an activator (11, 17, 49) and repressor (46, 47, 50) of NF-κB. Based on our findings, we conclude that physiological reconstitution of zinc represses immune activation, whereas a lack of zinc, in the setting of severe infection, leads to a systemic increase in NF-κB activation. Knowing that multilayered controls exist to keep NF-κB signaling in check, such as the autoregulatory feedback loop that involves the ubiquitin-editing protein A20, it is plausible that control over this or similar signaling axes may have been compromised by zinc deficiency (44). Interestingly, we observed an increase in A20 expression as early as 2 h following CLP treatment showing that nutrient levels (44).

Zinc deficiency enhances the expression of NF-κB-responsive genes 

**DISCUSSION**

**Fig. 8. Zinc deficiency enhances the expression of NF-κB-responsive genes in primary human lung epithelial cells.** A: primary human lung epithelial cells (hLECs) were cultured onto collagen-coated, semipermeable membranes. TPEN (20 μM) or zinc (5 μM) were used to modify zinc status as previously described. Cells were then stimulated with TNFα (10 ng/ml) for 6 h to activate the NF-κB pathway. Total RNA was isolated, and the expression of NF-κB target genes was measured. B: in addition, culture supernatants were obtained and measured to determine IL-6 and ICAM-1 concentrations (total donor number: n = 3; *Zn-/CLP vs. Ctrl/CLP, P < 0.05; †Zn+/CLP vs. Zn-/CLP, P < 0.05). MnSOD, manganese superoxide dismutase.

**Fig. 9. Zinc deficiency increases caspase-3 activity in the lung in response to CLP.** Identical lung specimens as described previously were processed and subjected to analysis for caspase-3 activity at 24 h after CLP in response to zinc deficiency as well as supplementation (n = 5 per group; *Zn-/CLP vs. Ctrl/CLP, P < 0.05; †Zn+/CLP vs. Zn-/CLP, P < 0.05.)
NF-κB activation. Whether our observations are due to changes in zinc-mediated signal activation or due to a lack of NF-κB suppression remains to be determined.

In the context of sepsis, it is well-accepted that excessive inflammation causes organ dysfunction. Relative to our findings, the acute respiratory distress syndrome (ARDS), a pathological condition induced by sepsis that adversely affects the lungs (1), occurs, in part, due to activation of the NF-κB pathway (3). In support of this, it has been shown that NF-κB binding activity in nuclear extracts from peripheral blood mononuclear cells of septic patients distinguished survivors from nonsurvivors (5, 11, 32). Specifically, increased and prolonged NF-κB activation correlated with increased morbidity and mortality. Consistent with this, we observed an increase in NF-κB activity in the lung of Zn-/CLP mice. Further inspection revealed that this occurred in both immune cells, presumably monocyte/macrophages and parenchymal cells. At the same time, we observed a significant increase in perivascular edema, consistent with epithelial damage, TUNEL-positive cells (27), and caspase-3 activity (Fig. 9), all of which are consistent with a pattern of acute lung injury as that observed in ARDS. Furthermore, we consistently observed an increase in the expression of IkBα and IkB family member B cell lymphoma 3 (Bcl-3), cytokines (TNFα, IL-1β), chemokines (CCL3, CXCL14), adhesion molecules (ICAM-1), and acute phase response genes (SAA1/2) in the lung. Taken together, these findings suggest that local dysregulation of NF-κB-mediated signaling, as a consequence of zinc deficiency, increases the incidence of lung injury, thereby contributing to a worse prognosis. How this occurs despite a concomitant increase in the expression of counterregulatory factors (IkBα and Bcl-3) remains unclear and requires further investigation.

We consistently observed a reduction of NF-κB activity following short-term zinc supplementation in septic mice simultaneously with increased survival and normalization of lung histology (27). This raises the question whether zinc supplementation in humans in a clinically relevant context (e.g., after the onset of sepsis) will provide benefit by preventing vital organ injury. A major limitation of our current investigation is that zinc supplementation is administered before sepsis, a scenario that would be difficult to recapitulate in a clinical setting unless large populations were screened for zinc deficiency, an impractical scenario at the present time. However, given the projected high incidence of zinc deficiency a priori in sepsis patients, further studies that explore zinc supplementation after the onset of sepsis are warranted. This hypothesis is supported by previous animal studies demonstrating that systemic zinc administration during or shortly after the onset of infection decreases mortality (39, 42). It is important to identify that, in these studies, animals were zinc-sufficient before infection and that supraphysiological zinc doses obviated in any benefit and may have increased mortality. Clearly, it will be important to determine whether the therapeutic advantages of zinc administration outweigh risk and, if so, identify which patients will benefit most from zinc supplementation.

Hypozincemia is one of the first systemic changes observed in sepsis that currently is believed to be a consequence of zinc redistribution from the blood compartment into the liver, and perhaps other vital organs (30), to protect the host against pathogen invasion. Gaetke et al. (13) observed an acute decrease of serum zinc levels in response to systemic LPS administration to healthy adult volunteers, which is similar to what we observed in septic mice on control diets (data not shown). A concomitant increase in the expression of the acute phase response protein SAA1/2 was also observed. Considering that the expression of SAA1/2 was further increased in the lung of Zn-/CLP mice compared with normal dietary counterparts (Fig. 5), we conclude that nutritional zinc status also has a profound impact on the acute phase response in vivo.

In summary, this investigation builds on our previous work and others showing that zinc deficiency is detrimental to the host through dysregulation of the initial host response to systemic infection by enhancing inflammation, organ damage, and mortality. Based on our observations using animal and cell culture models, we consistently demonstrate that zinc deficiency results in excessive activation of the NF-κB pathway in a sepsis model and that the resulting consequences are augmentation of the innate immune and acute phase response systemically and within the lung. These effects were largely reversed following zinc supplementation, which normalized inflammatory balance through reduction of NF-κB signaling. Based on these observations, future studies are proposed to explore the incidence of zinc deficiency in “at-risk” subjects as well as rational therapeutic strategies aimed at providing zinc supplementation.

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REFERENCES
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