Hepatic Hepcidin Protects against Polymicrobial Sepsis in Mice by Regulating Host Iron Status

CongLi Zeng, M.Sc., QiXing Chen, Ph.D., Kai Zhang, M.D., QingHua Chen, M.Sc., ShengWen Song, M.D., XiangMing Fang, M.D.

ABSTRACT

Background: Hepcidin is a master regulator of iron metabolism primarily produced by the liver. Markedly increased hepcidin levels have been observed in septic individuals, while decreased hepatic hepcidin expression has been demonstrated in liver diseases that tend to develop into sepsis. However, the role of liver hepcidin in sepsis remains unknown.

Methods: Mouse hepatic hepcidin expression was silenced using adenovirus-mediated hepcidin-specific short hairpin RNA injected *via* the tail vein. Sepsis was induced by cecal ligation and puncture, and the outcome (n = 23 for hepcidin knockdown mice, n = 15 for controls) and pathogenic changes (n = 5) related to sepsis were evaluated. The impact of alteration of iron status on the survival rate of hepatic hepcidin knockdown mice (n = 18 to 19) was also investigated.

Results: Disruption of liver hepcidin expression increased serum iron level (537.8 ± 28.1 μg/dl [mean ± SD] *vs.* 235.9 ± 62.2 μg/dl; $P < 0.05$) and reduced iron content in the spleen macrophages at the steady state. Hepatic hepcidin knockdown mice not only showed increased 7-day mortality (73.9% *vs.* 46.7%; $P < 0.05$), but also had exacerbated organ damage and oxidative stress, as well as compromised host inflammatory responses and bacterial clearance at 24 h after polymicrobial sepsis. Treating the hepatic hepcidin knockdown mice with low-iron diet plus iron chelation decreased systemic iron content (serum level: 324.0 ± 67.4 μg/dl *vs.* 517.4 ± 13.4 μg/dl; $P < 0.05$) and rescued the mice from lethal sepsis (7-day survival: 36.8% *vs.* 83.3%; $P < 0.01$).

Conclusions: Hepatic hepcidin plays an important role in sepsis through regulation of iron metabolism. The findings may have potential therapeutic implications for liver diseases in which hepcidin expression is decreased. **(Anesthesiology 2015; 122:374-86)**

SEPSIS is a systemic inflammatory reaction syndrome that occurs concomitantly with a pathologic infection; it is considered severe when associated with acute organ dysfunction.[1,2] More than 750,000 new cases of sepsis are reported annually in the United States, and the mortality rate of patients with sepsis ranges from 30 to 70% worldwide.[3–5] Sepsis is a leading cause of mortality in critical illness throughout the world, with tremendous international economic and social burden.[3,5,6]

The pathogenesis of sepsis is complex. Many different molecules, cells, and organs are involved in the development of this syndrome.[1,7] The liver, which actively modulates the inflammatory process by scavenging bacteria, inactivating bacterial products, and clearing inflammatory mediators that play a major role in host defense mechanisms, is a key organ in sepsis. The liver also synthesizes many acute-phase proteins in response to infection and inflammation.[8,9] This "The work has been partly presented in abstract form at the 2013 Annual Meeting…" orchestrated response is regulated by the paracrine or remote release of mediators, such as interleukin (IL)-6. These mediators affect subsets of acute phase genes through acting on a transcriptional or posttranscriptional level, and result in an up-regulation or down-regulation of proteins involved in the pathophysiology of sepsis.

What We Already Know about This Topic

- Markedly increased hepcidin levels have been observed in septic patients, while decreased hepatic hepcidin expression has been demonstrated in liver diseases that tend to develop into sepsis.

What This Article Tells Us That Is New

- Disruption of mouse hepatic hepcidin expression led to significant increases in organ damage and mortality when sepsis was produced. Decreasing the iron levels in these mice improved survival.

Some of the acute phase proteins have been shown to not only contain pathogens and limit the inflammatory cascade, but also control damage and contribute to the repair of tissue during sepsis.[10–12] However, the role of many other acute-phase reactants in sepsis remains unknown.

Hepcidin is a β-defensin–like antimicrobial peptide that is primarily produced by the liver; it is a type II acute-phase protein.[13,14] Hepcidin acts as an important iron regulatory hormone. Its synthesis is finely regulated; it can be induced by iron overload and inflammation and suppressed by iron deficiency, hypoxia, and anemia.[15,16] Inappropriate synthesis of hepcidin has been shown to be involved in the pathogenesis of iron-related metabolic disorders.[17–19] Mice overexpressing

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hepcidin are severely anemic at birth,[20] and hepcidin deficiency has been demonstrated to be the primary cause of most forms of hereditary hemochromatosis and β-thalassemia.[21–24] In addition to its iron regulatory activity, hepcidin modulates lipopolysaccharide-induced acute inflammatory responses and protects mice from lipopolysaccharides-induced endotoxemia.[25] Furthermore, markedly increased hepcidin levels have been observed in urine from septic donors and sera of septic patients.[26,27] These findings suggest that hepcidin may participate in the pathogenesis of sepsis. However, the details of its function in sepsis remain unclear.

The mouse genome contains two highly similar hepcidin genes, *HEPC1* and *HEPC2*. *HEPC1* codes for a peptide with biological activity similar to that of human hepcidin.[28] We hypothesized that hepcidin plays critical roles in the pathogenesis of sepsis. Using a hepatic *HEPC1* knockdown murine model, we investigated the impact of disruption of hepcidin on mortality and organ damage as well as on bacterial clearance and cytokine production in cecal ligation and puncture (CLP)–induced sepsis. The iron metabolic status of the animals was also measured, and the effect of regulating iron status on macrophage function and on the survival of septic mice was further assessed.

Materials and Methods

Animals

Male BALB/c mice (weight 20–25 g) were purchased from the animal center of Zhejiang Chinese Medicine University (Hangzhou, China). The animals were maintained on standard chow and tap water, which were available *ad libitum*, in a temperature-controlled chamber at 24°C with a 12-h light–dark cycle. The mice were randomly assigned to experimental condition throughout the study. The animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

Adenoviral Vector-mediated Hepcidin-specific Short Hairpin RNA

Anti-mouse *HEPC1* small interfering RNA (*ACAGAUGAGACAGACUACA*dTdT), which was described previously,[29] was converted to short hairpin RNA (ACCG*ACAGATGAGACAGACTACA*TTCATGAGA *TGTAGTCTGTCTCATCTGT*CTTTTT) (Invitrogen, Shanghai, China). Adenovirus plasmids containing the enhanced green fluorescent protein gene and *HEPC1* short hairpin RNA or a non-specific control oligonucleotide (GCCTAAGGTTAAGTCGCCCTCGCGAACG AAGGCGAGGGCGACTTAACCTTAGGTT) were constructed, and adenoviruses were prepared by Life Technologies (Shanghai, China). The recombinant and control viruses were named Ad-shHepc1 and Ad-shNeg, respectively. Viral titer determination (infectious units/ml) was performed using a tissue culture infectious dose of 50. Viral stocks with titers ranging from 10^{11} to 10^{12} infectious units/ml were used in the experiments.

Polymicrobial Sepsis Model

Ad-shHepc1 or control Ad-shNeg was administered to male BALB/c mice by hydrodynamic injection *via* the tail vein.[30] On the 13th day after administration, the mice underwent CLP surgery or sham operation as described previously.[31] The sample sizes were chosen based on previous experience. After surgery, the mice were resuscitated by subcutaneous injection of sterile saline and given free access to food and water. In some experiments, Ad-shHepc1 administrated mice received a low-iron diet in combination with deferoxamine (Sigma-Aldrich, St. Louis, MO) treatment. In these cases, after the administration of recombinant adenovirus, the mice were fed a low-iron diet (4 ppm) until the day of CLP surgery and intraperitoneally injected with deferoxamine (100 mg kg^{-1} day^{-1}) beginning 7 days before CLP surgery. Control mice were maintained on a standard rodent diet and injected with the same volume of normal saline. The survival of mice subjected to CLP was monitored for 7 days.

Immunofluorescent Histochemical Staining

Liver tissues were fixed for 24 h in 4% paraformaldehyde-buffered solution, paraffin embedded and sectioned at 4 μm. After deparaffinization and hydration, the sections were stained with 4′,6-diamidino-2-phenylindole and then observed under a fluorescence microscope.

Real-time Polymerase Chain Reaction

Total RNA was extracted from the harvested tissues (liver, spleen, lung, kidney and peripheral leukocytes) using TRIzol® reagent (Invitrogen, Carlsbad, CA). Complementary DNA was generated by reverse transcription of 1 μg of total RNA using the Reverse Transcription System (Promega, Madison, WI). Hepcidin messenger RNA (mRNA) levels were quantified by real-time polymerase chain reaction (PCR) using the standard SYBR® Green PCR protocol on a CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA). The 10-μl PCR volume contained 5 μl iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc.), 1 μl of each primer and 1 μl complementary DNA. The reaction conditions were as follows: 3 min at 95°C followed by 40 cycles of 95°C for 10 s, 50°C for 30 s, and 72°C for 30 s. Each sample was assayed in triplicate. The housekeeping gene β-actin was used as an internal control. Gene expression levels were determined using the $2^{-\Delta\Delta CT}$ relative quantification method.

Immunohistochemical Analysis

Liver tissues and other organs (spleen, lung, and kidney) from the experimental mice were fixed for 24 h in a 4% paraformaldehyde-buffered solution and paraffin embedded. To measure the hepcidin levels, the samples were sectioned in 4-μm slices and subjected to a standard immunohistochemical procedure using a primary antibody from rabbit (Abcam, San Francisco, CA) and a related secondary antibody (ZSGB-BIO, Beijing, China). Evaluation of the immunohistochemical staining

was performed using an Olympus BX-50 light microscope (Olympus, Tokyo, Japan). Five images per sample were randomly photographed using a modular photomicrographic system attached to the microscope. Stain density was analyzed using the Image Pro-Plus 6.0 analysis system (Media Cybernetics Inc., Silver Spring, MD); the integral optical density was determined as the hepcidin level.

Bacterial Inspection

For bacterial colony-forming unit (CFU) analysis, blood and tissue homogenates of liver, lung, and spleen from the experimental animals were serially diluted in phosphate buffered saline and used to inoculate agar-medium plates. After incubation at 37°C for 16 to 24 h, CFUs were counted and expressed as $\log_{10}$ of CFU/ml of blood or $\log_{10}$ of CFU/g of tissue.

Histological Evaluation

The liver and lung tissues were fixed for 24 h in a 4% paraformaldehyde-buffered solution, embedded, and sectioned at 3 μm. Histological examination was conducted blindly after staining with hematoxylin and eosin. The lung injury scores were assessed as previously described.[32]

Liver Function Test

The serum samples were obtained from the experimental mice, and aspartate aminotransferase and alanine aminotransferase levels were detected using commercially available kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling Assay

To detect apoptotic cell death in the spleens of the experimental mice, terminal deoxynucleotidyl transferase dUTP nick-end labeling assays were performed using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Briefly, slides containing spleen tissues were incubated with terminal deoxynucleotidyl transferase dUTP nick-end labeling reaction mixture for 1 h at 37°C. After washing with phosphate buffered saline, the slides were incubated with peroxidase-conjugated antibody for 30 min at 37°C and developed using a 3,3′-diaminobenzidine system. Apoptotic cells were counted in every high-power field (400×) under microscopy, and five fields were randomly selected from each sample.

Serum Iron, Liver Iron, and Spleen Iron Determinations

Blood was collected from the experimental mice and kept for 1 h at room temperature. Serum was then obtained by centrifugation. Serum iron concentration were measured by atomic absorption spectroscopy.[33] Nonheme iron concentrations in the liver and spleen tissues were determined as previously described.[34] Spleen iron content was also determined by Prussian blue staining using a commercially available kit according to the manufacturer's instructions (Shanghai Yuanye Bio-Technology Co., Shanghai, China).

Nicotinamide Adenine Dinucleotide Phosphate Oxidase Activity

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was determined by the lucigenin-enhanced chemiluminescence method as previously described.[35,36] The activation of NADPH oxidase could oxidize NADPH into NADP, and then oxygen molecules get electrons to form superoxide anion O_2^-. The superoxide anion O_2^- could transmit the electrons to lucigenin and make lucigenin reduced which then release energy that could be detected by luminometer. The more energy released by lucigenin is detected, the more activity of NADPH oxidase is estimated. To perform this assay, the liver tissues were prepared into homogenate. The activity of NADPH oxidase was measured by a luminescence assay with 5μM lucigenin (Sigma-Aldrich) as the electron acceptor and 0.1mM NADPH (Sigma-Aldrich) as the substrate. The emitted luminescence was detected by a luminometer (Thermo Fisher Scientific Inc., Waltham, MA), and buffer blank was subtracted from each reading. The activity of NADPH oxidase was expressed as arbitrary units/mg of protein.

Reactive Oxygen Species Production

The liver tissue was prepared into single cell suspension digested by collagenase IV (Life Technologies, Grand Island, NY) and DNase (Sangon Biotech Co., Shanghai, China). The cell suspension was first stained by F4/80 antibody and Gr-1 antibody (eBioscience, San Diego, CA) for 15min, respectively. For measuring total intracellular oxidative stress,[37] the cells were treated with fluorogenic probes CellROX® Deep Red Reagent (Life Technologies) at a final concentration of 5 μM and incubated for 30 min at 37°C. The cells were then washed three times with phosphate buffered saline, and analyzed by flow cytometer using 640/665 nm excitation/emission filters. Reactive oxygen species (ROS) level was reported as median fluorescent intensity.

Enzyme-linked Immunosorbent Assay

Serum tumor necrosis factor alpha (TNF-α) and IL-6 concentrations were measured using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Abcam).

Phagocytosis Assay

To investigate the effect of intracellular iron concentration on macrophage phagocytosis, RAW264.7 cells were incubated with various concentrations of desferoxamine. Twenty-four hours later, RAW264.7 cells were incubated with fluorescent polystyrene microspheres (Life Technologies) for 2 h. After 3 washes with phosphate-buffered saline, the fluorescence was observed under a fluorescence microscope and detected by flow cytometer.

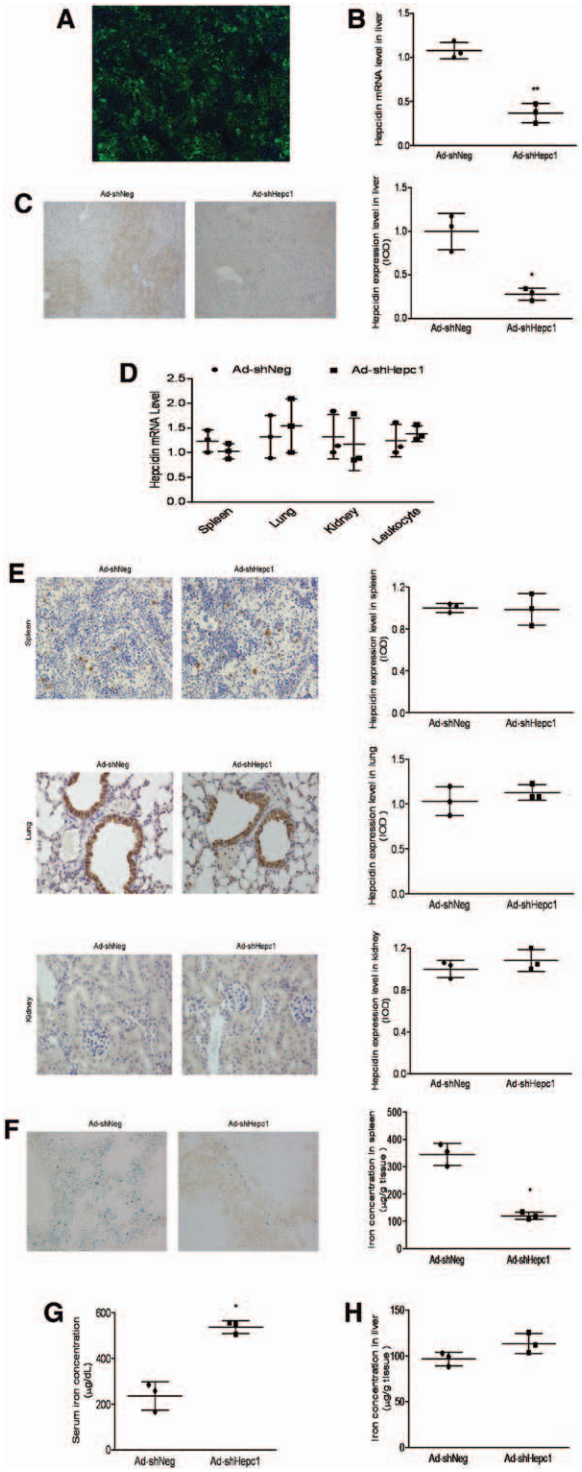

Fig. 1. Hepcidin expression and iron status in the hepatic hepcidin knockdown mouse model. Mice were treated with Ad-shHepc1 or Ad-shNeg through hydrodynamic injection *via* the tail vein. (*A*) On the 5th day after administration of Ad-shHepc1, the liver tissue was stained with 4′,6-diamidino-2-phenylindole and observed under a fluorescence microscope (n = 2). (*B*, *C*) On the 13th day after administration of Ad-shHepc1 or Ad-shNeg, hepcidin expression levels in the liver were assessed by real-time PCR and immunohistochemistry (n = 3). (*D*, *E*) Hepcidin levels in other tissues and cells were also quantified by real-time PCR and immunohistochemistry (n = 3). (*F*) Iron content in the spleen was evaluated by Prussian blue staining and quantified as microgram per gram wet tissue after digestion with acid buffer respectively (n = 3). (*G*) Serum iron level was determined using atomic absorption spectroscopy (n = 3). (*H*) Iron concentrations in the liver tissue were measured after digestion with acid buffer and expressed as microgram per gram wet tissue (n = 3). **P* < 0.05; ***P* < 0.01. IOD = integral optical density; mRNA = messenger RNA; PCR = polymerase chain reaction.

Macrophage Inflammatory Response

To study the impact of intracellular iron content on the macrophage inflammatory response, RAW264.7 cells were treated with various concentrations of deferoxamine for 24 h and then stimulated with 100 ng/ml lipopolysaccharides. Six hours later, the cells were collected, and the levels of mRNA for the inflammatory cytokines TNF-α and IL-6 were detected by quantitative real-time PCR as described in section "*Real-Time Polymerase Chain Reaction*" of Materials and Methods.

Statistical Analysis

Data are expressed as the mean ± SD or median (interquartile range) where applicable. Comparisons of differences in continuous variables were conducted with Student *t* test or Mann–Whitney U test or one-way ANOVA test with Bonferroni correction for multiple comparisons where appropriate. Survival curves (Kaplan–Meier plots) were compared using the log-rank test. Statistical analysis was performed using GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA). A two-tailed value of *P* less than 0.05 was considered to be statistically significant.

Results

Knockdown of Hepcidin in Liver and Its Effect on Iron Homeostasis

To study the role of hepatic hepcidin in sepsis, we developed a mouse model to knock down hepatic hepcidin through hydrodynamic injection of Ad-shHepc1 *via* the tail vein. To confirm that the recombinant adenovirus was successfully transduced *in vivo*, the enhanced green fluorescent protein marker was assayed on the 5th day after injection. As expected, green fluorescence was observed in the liver (fig. 1A) but not in other organs (data not shown). Furthermore, on the 13th day after administration of adenovirus, hepcidin levels in the livers of the Ad-shHepc1 challenged mice were significantly lower than those in the control mice, as demonstrated by real-time quantitative PCR and immunohistochemistry (fig. 1, B and C). However, the mRNA level and protein level of hepcidin in other tissues (spleen, lung, kidney and peripheral leukocytes) were comparable in the two groups (fig. 1, D and E). Considering the master function of hepcidin in regulating iron homeostasis and the role of iron in the host immune response,[19,38,39] the effect

of interfering liver hepcidin expression on iron homeostasis was also measured. In spleen tissue which was rich for macrophages, the iron content was significantly lower in the hepcidin knockdown mice than that in the control mice, as determined by Prussian blue staining and quantified after tissue digestion with acid buffer (fig. 1F), indicating that a large amount of iron was released from spleen macrophages in these mice. Consistent with this finding, serum iron levels in the hepcidin knockdown mice were significantly higher than that in the control mice (fig. 1G). In addition, in liver tissues, the iron content had a trend to be higher in the hepcidin knock-down mice (fig. 1H).

After CLP challenge, significantly lower hepcidin levels were also observed in the liver of the Ad-shHepc1-treated mice (fig. 2, A and B), but not in other tested tissues (fig. 2C). The iron content in the spleen and serum iron levels between the two groups after CLP surgery showed comparable patterns to those in the steady state (fig. 2, D and E).

Effect of Hepatic Hepcidin on Mortality and Organ Damage in Polymicrobial Sepsis

To investigate the role of hepatic hepcidin in sepsis, the impact of hepcidin silence on the outcome of sepsis was studied. As shown in figure 3A, most of the hepcidin knockdown mice succumbed to sepsis within 72 h of CLP surgery, while 53.3% of the mice in the control group were resistant to sepsis and survived for 7 days. The extent of organ damage in the experimental mice was evaluated at 24 h after CLP or sham operation. Hematoxylin and eosin staining revealed large areas of necrosis in the livers of hepcidin knockdown mice, and serum levels of aspartate transaminase and alanine aminotransferase as biochemical markers of liver dysfunction in these animals were significantly higher than those in the controls (fig. 3B). Compared to the control mice, the hepcidin knockdown mice experienced more severe pulmonary injury after CLP as assessed by lung injury scores (fig. 3C). To test the severity of spleen damage after sepsis, lymphocyte apoptosis in the spleen tissue was assessed using the terminal deoxynucleotidyl transferase dUTP nick-end labeling method. The hepcidin knockdown mice had more apoptotic lymphocytes than the control mice after CLP (fig. 3D).

Effect of Hepatic Hepcidin on Systemic Inflammatory Response and Bacterial Burden in Polymicrobial Sepsis

To examine the systemic inflammatory response, serum concentrations of pro-inflammatory cytokines were measured 24 h after CLP. IL-6 levels in the hepcidin knockdown mice were significantly lower than those in control mice, whereas TNF-α levels were comparable in the two groups (fig. 4A). These results suggest that the inflammatory response that occurs during CLP-induced polymicrobial sepsis may be dampened in the hepcidin knockdown mice.

We next measured bacterial burden at 24 h after CLP. Compared to the control mice, the hepcidin knockdown mice showed markedly higher bacterial CFUs in the blood (fig. 4B). Similarly, the bacterial loads in the livers and lungs of the hepcidin knockdown mice were significantly higher than those in control mice (fig. 4B). An increased bacterial burden in the spleen of the knockdown group was also observed, although a significant difference was absent (fig. 4B). These observations suggest that hepcidin participates in the pathogenesis of polymicrobial sepsis.

Effect of Hepatic Hepcidin on Oxidative Stress Status in Polymicrobial Sepsis

Accumulated evidence shows that excess iron increases the generation of ROS and the peroxidation of membrane lipids,

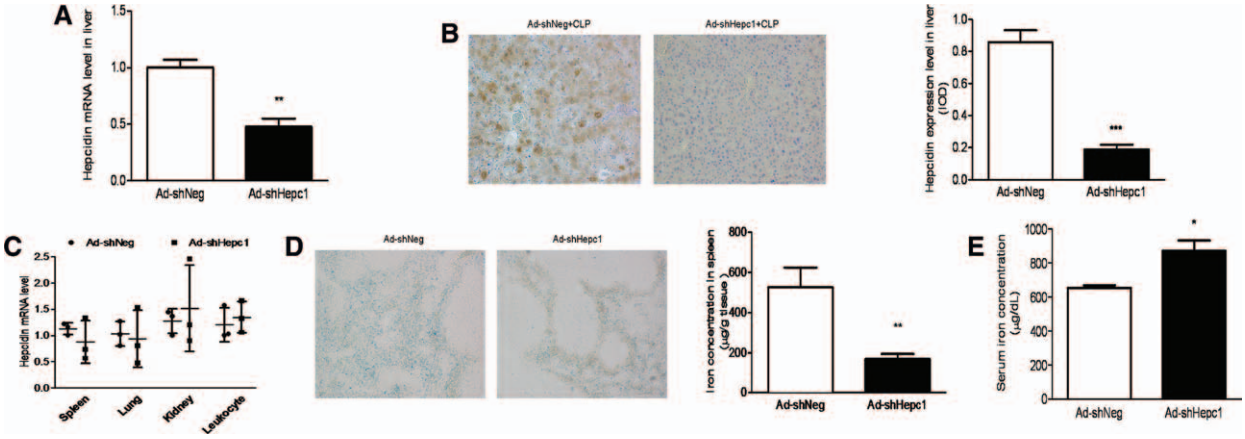

Fig. 2. Hepcidin expression and iron status in the hepatic hepcidin knockdown mouse model 24 h after CLP. On the 13th day after administration of Ad-shHepc1 or Ad-shNeg, mice underwent CLP surgery. (*A*, *B*) Twenty-four hours later, liver hepcidin expression levels were quantified by real-time PCR and immunohistochemistry (n = 5). (*C*) The levels of hepcidin in other tissues and cells were also measured by real-time PCR (n = 3). (*D*) The iron content in the spleen was evaluated by Prussian blue staining and quantified as microgram per gram wet tissue after digestion with acid buffer (n = 5). (*E*) Serum iron concentrations were determined using atomic absorption spectroscopy (n = 5). **P* < 0.05; ***P* < 0.01; ****P* < 0.001. CLP = cecal ligation and puncture; IOD = integral optical density; mRNA = messenger RNA; PCR = polymerase chain reaction.

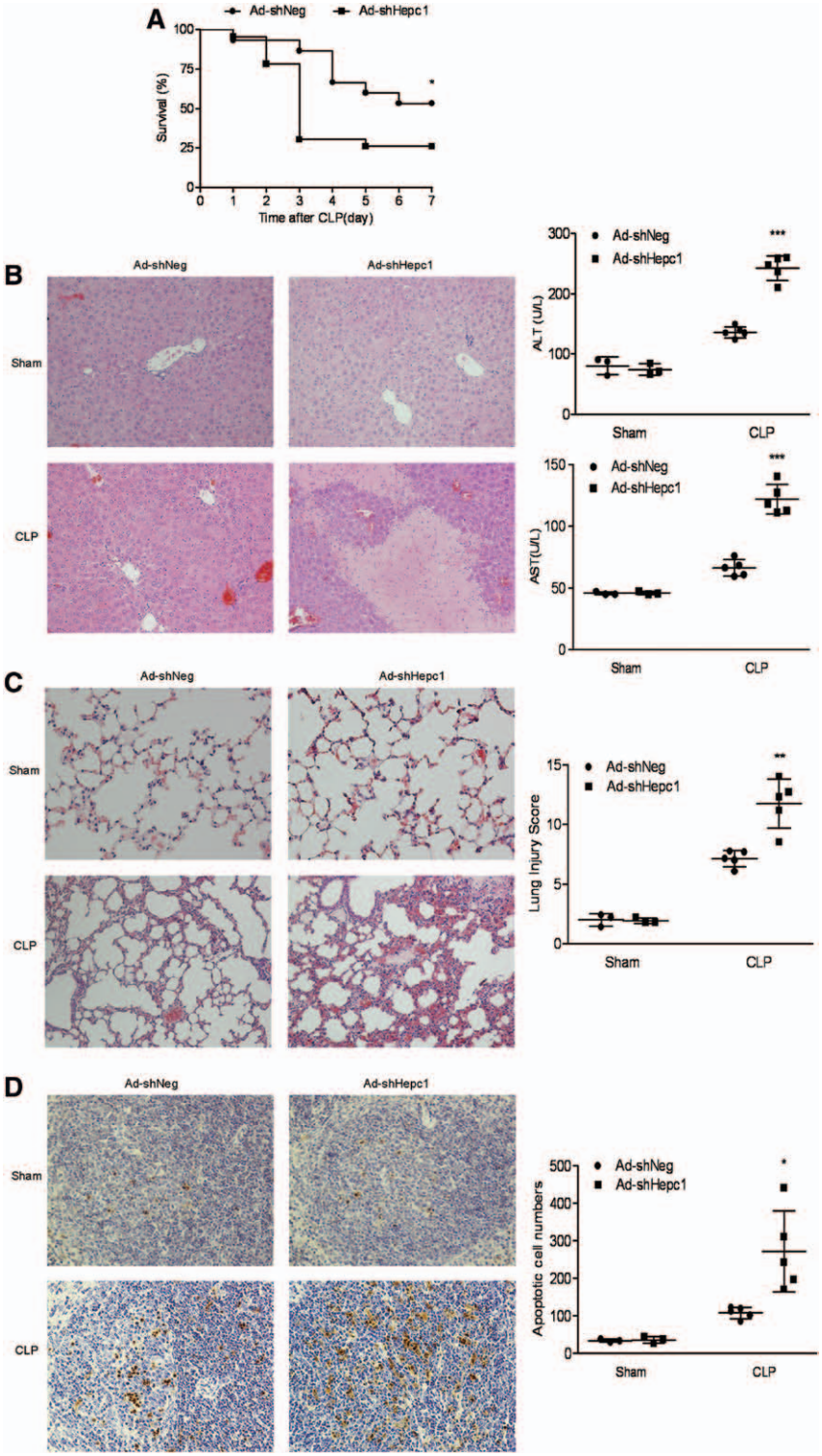

Fig. 3. Hepatic hepcidin knockdown mice show higher mortality and more severe organ damage during polymicrobial sepsis. On the 13th day after administration of Ad-shHepc1 or Ad-shNeg, the mice underwent CLP surgery. Tissues were harvested from both groups 24 h after CLP and Sham operation. (*A*) The 7-day survival rate was assessed (Ad-shHepc1 group, n = 23; Ad-shNeg group, n = 15). (*B*) The liver tissues were stained by hematoxylin and eosin, and the serum transaminase (ALT and AST) were measured (n = 5). (*C*) The lung tissues were stained by hematoxylin and eosin, and the injury scores were evaluated (n = 5). (*D*) The spleen tissues were stained by TUNEL, and the number of apoptotic cell per high power field was counted (n = 5). **P* < 0.05; ***P* < 0.01; ****P* < 0.001. ALT = alanine aminotransferase; AST = aspartate aminotransferase; CLP = cecal ligation and puncture; TUNEL = TdT-mediated dUTP nick end labeling.

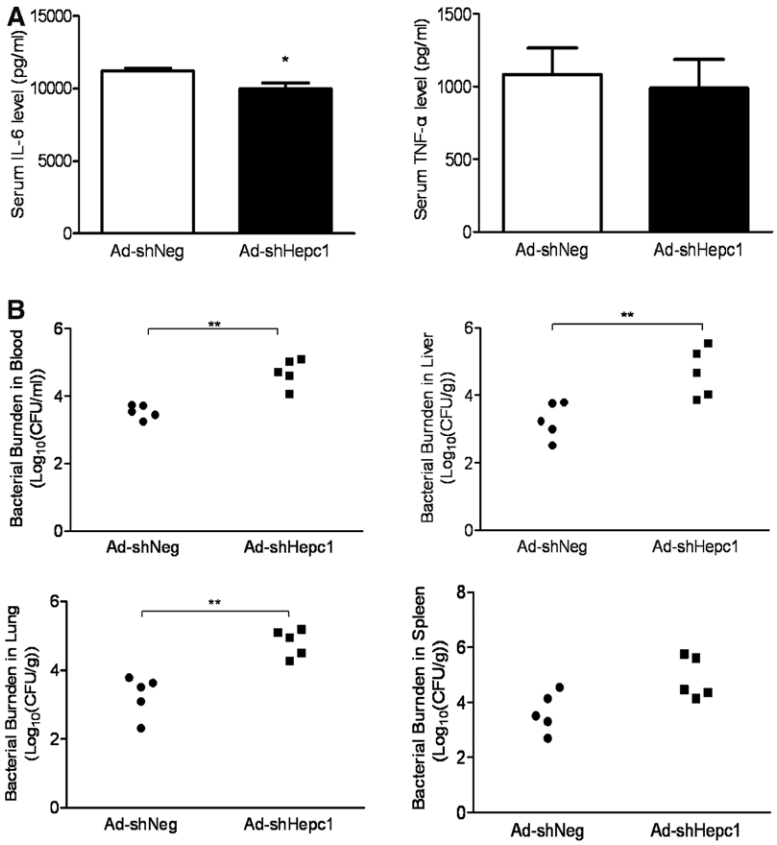

Fig. 4. Hepatic hepcidin knockdown mice show a dampened inflammatory response and higher bacterial burden. Samples were collected 24 h after CLP. (*A*) The serum levels of IL-6 and TNF-α were detected (n = 5). (*B*) Bacterial loads in blood, liver, lung, and spleen were determined, and the results are expressed as $\log_{10}$(CFU/ml blood) in blood and $\log_{10}$(CFU/g tissue) in the organs (n = 5). *$P < 0.05$; **$P < 0.01$. CFU = colony forming units; CLP = cecal ligation and puncture; IL-6 = interleukin 6; TNF-α = tumor necrosis factor-alpha.

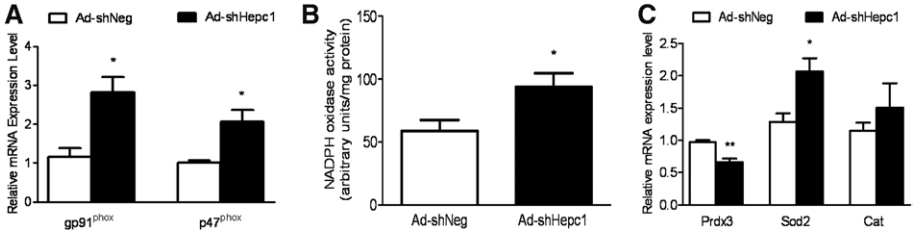

Fig. 5. Hepatic hepcidin knockdown mice show enhanced oxidative status 24 h after CLP. (*A, B*) The mRNA levels of NADPH oxidases and the activity of NADPH oxidases in the liver were measured as described in the Materials and Methods section (n = 5). (*C*) The mRNA levels of antioxidant proteins in the liver were also measured (n = 5). *$P < 0.05$; **$P < 0.01$. Cat = catalase; CLP = cecal ligation and puncture; mRNA = messenger RNA; NADPH = nicotinamide adenine dinucleotide phosphate; Prdx3 = peroxiredoxin 3; SOD = superoxide dismutase.

leading to cell and organ damage.[40,41] We therefore investigated whether the altered iron status of hepcidin knockdown mice influenced the activity of NADPH oxidases, a family of enzymes that are specifically involved in the production of ROS. After CLP, the mRNA levels of gp91phox and p47phox, the two main subunits of the NADPH oxidase complex, and the NADPH oxidase activity, were significantly increased in the liver of the hepcidin knockdown mice (fig. 5, A and B). The transcription levels of antioxidant proteins, including peroxiredoxin 3, manganese superoxide dismutase and catalase, were also measured. In hepatic hepcidin knockdown mice, the level of peroxiredoxin 3 mRNA was decreased, while manganese superoxide dismutase and catalase mRNA levels were markedly increased (fig. 5C).

Effect of Intracellular Iron Status on Macrophage Function

Because knockdown of hepcidin resulted in the release of iron from macrophages, we next examined whether low

intracellular iron concentrations influence macrophage functions such as phagocytosis, inflammatory response and ROS production. RAW264.7 cells, a murine macrophage cell line, were treated with various concentrations of desferoxamine to chelate intracellular iron and then incubated with fluorescence-labeled polystyrene microspheres. Phagocytosis was assessed by fluorescence microscopy (fig. 6A) and flow cytometry (fig. 6B). As expected, the phagocytic index and mean fluorescent intensity in desferoxamine-treated macrophages were lower than those in the untreated macrophages, suggesting a decreased ability to phagocytize in the macrophages with low intracellular iron level.

Furthermore, under lipopolysaccharides challenge, the inflammatory response in desferoxamine-treated macrophages was rather limited, as evidenced by decreased mRNA levels of the cytokines IL-6 and TNF-α in these cells (fig. 6C).

Also, to link the iron status and functions of immune cells, ROS production in liver neutrophils and macrophages were measured. Neutrophil ROS production was significantly reduced in the hepatic hepcidin knockdown mice compared to that in the controls (fig. 6D). ROS production in hepatic macrophages from the hepcidin knockdown mice was also lower than that in the controls, although a significant difference was absent (fig. 6D). These results suggest that the disorder of iron homeostasis in the hepcidin knockdown mice leads to macrophage dysfunction against infection and inflammation.

Effect of Iron Status on Survival of Polymicrobial Sepsis in Hepatic Hepcidin Knockdown Mice

To further clarify the role of iron in the hepcidin knockdown mice during sepsis, the mice were treated with a low-iron diet plus deferoxamine or left untreated. After treatment, serum iron concentration, and hepatic iron level, as well

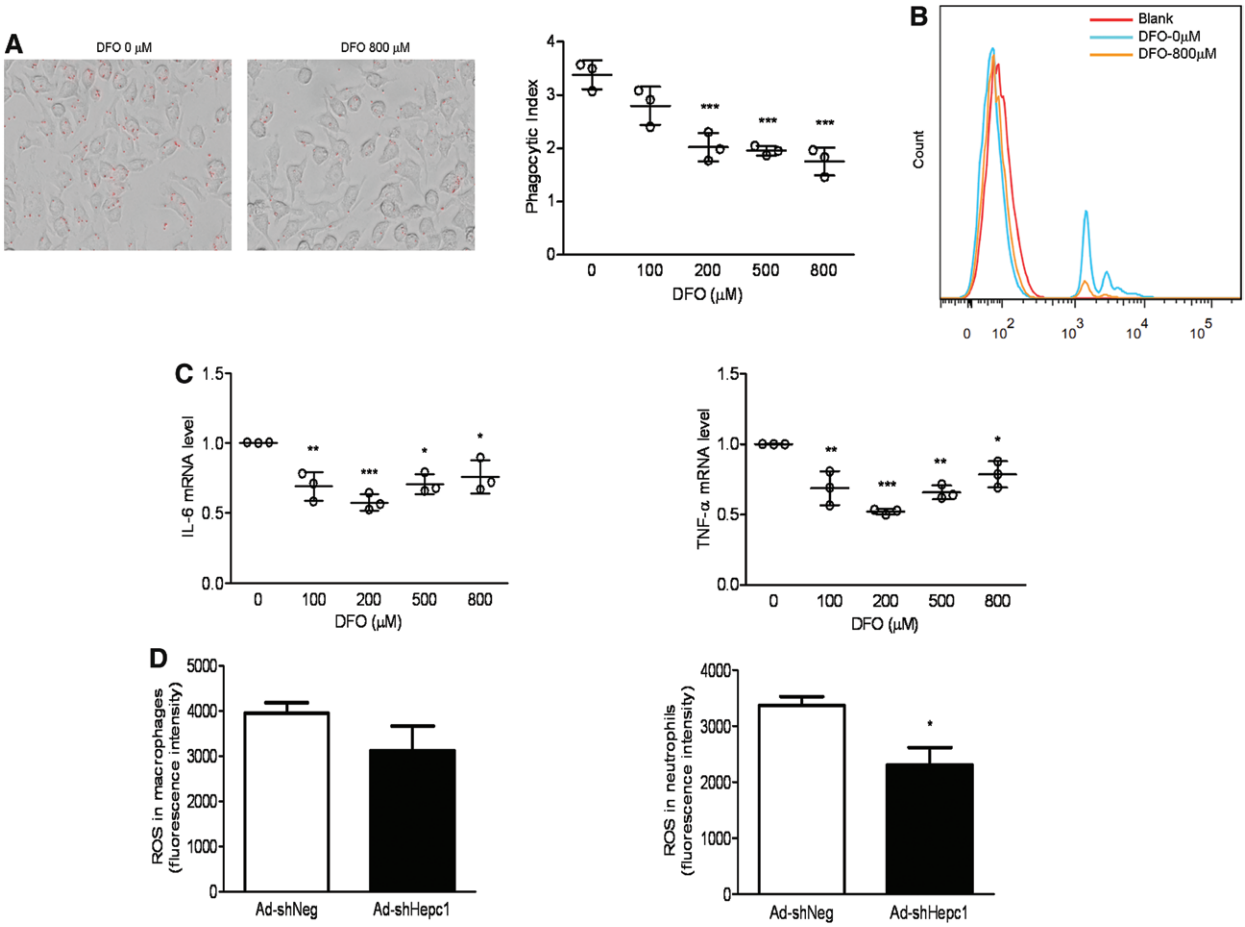

Fig. 6. The lower intracellular iron status influences macrophage functions. RAW264.7 cells were treated with various concentrations of desferoxamine (DFO) and incubated with microspheres or challenged with lipopolysaccharide. (*A, B*) Phagocytosis was assessed by fluorescence microscopy and flow cytometry (n = 3). Representative results with 800 μM desferoxamine treatment and without desferoxamine treatment were shown. (*C*) The mRNA levels of cytokines IL-6 and TNF-α in the desferoxamine-treated macrophages were measured using real-time PCR (n = 3). (*D*) Twenty-four hours after CLP, in Ad-shHepc1- or Ad-shNeg-treated mice, ROS production in liver macrophages and neutrophils was measured by flow cytometry (n = 5). **P* < 0.05; ***P* < 0.01; ****P* < 0.001. CLP = cecal ligation and puncture; IL-6 = interleukin 6; mRNA = messenger RNA; PCR = polymerase chain reaction; ROS = reactive oxygen species; TNF-α = tumor necrosis factor-alpha.

as spleen iron content in the hepcidin knockdown mice were significantly decreased (fig. 7, A–C). This treatment also led to a much lower IL-6 concentration when the mice were challenged with CLP surgery (fig. 7D). Furthermore, decreasing the systemic iron level significantly improved the 7-day survival of the hepatic hepcidin knockdown mice with polymicrobial sepsis (fig. 7E). This observation further demonstrates that hepatic hepcidin plays a critical role in sepsis through modulating iron metabolism.

Discussion

This study showed that disruption of hepatic hepcidin altered iron metabolic status in mice, leading to increased mortality and organ damage as well as compromised host inflammatory response and bacterial clearance during polymicrobial sepsis. Decreasing the systemic iron level rescued the hepatic hepcidin knockdown mice from lethal sepsis. These findings suggest that hepatic hepcidin plays an important role in sepsis.

Hepcidin is a type-II acute-phase protein that is predominantly expressed in hepatocytes.[13–15] Hepcidin is also expressed in other tissues and cell types including lung, spleen, and myeloid cells.[42] In liver, expression of hepcidin in response to inflammatory and infectious conditions is primarily mediated by the IL-6/signal transducer and activator of transcription 3 signaling pathway, while hepcidin expression in myeloid cells is dependent on the activities of Toll-like receptor 4 and nuclear factor kappa B.[43–45] The different mechanisms of transcriptional regulation of hepcidin in different cells suggest that this peptide may have diverse

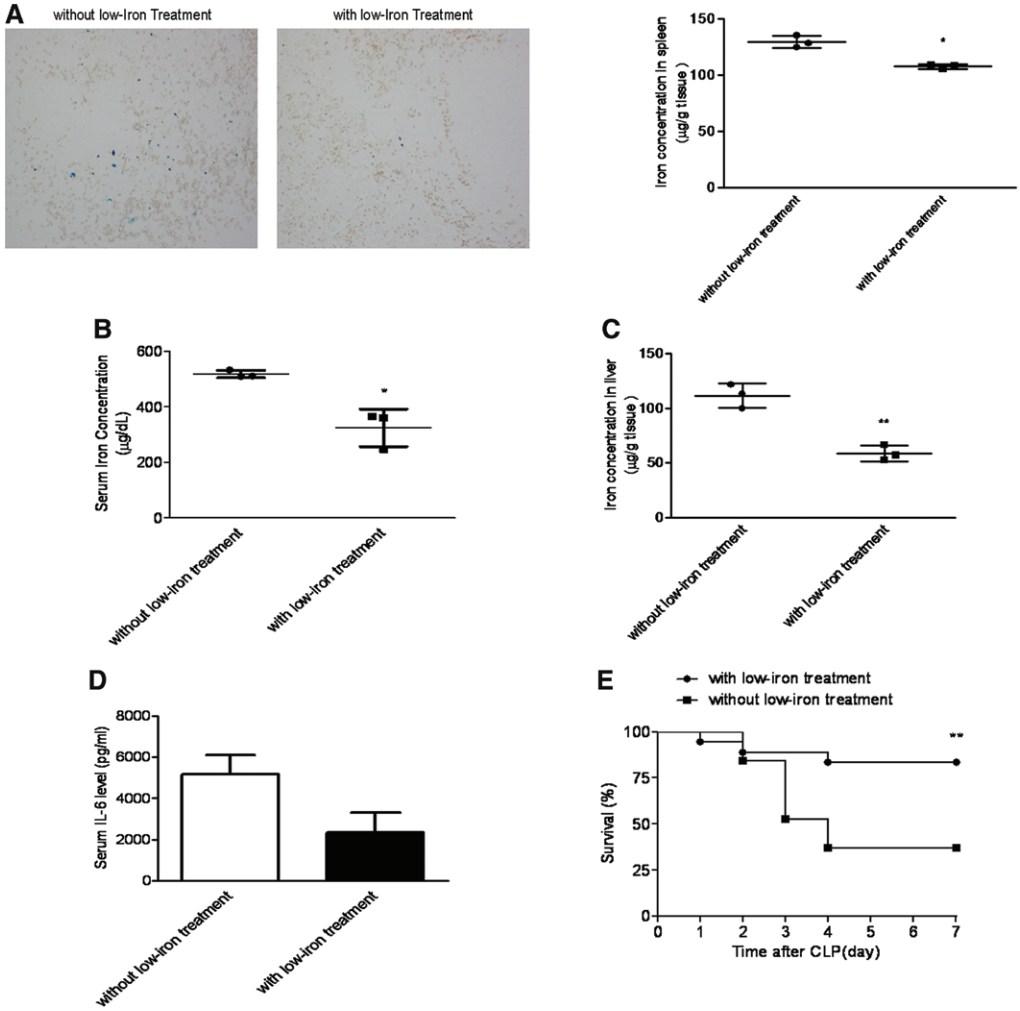

Fig. 7. Low-iron treatment improves iron status and sepsis survival in the hepatic hepcidin knockdown mice. After administration of Ad-shHepc1, the mice were given with or without low iron treatment. (*A–C*) On the 13th day, the iron status of mice was measured. (*A*) The iron content in the spleen was evaluated by Prussian blue staining and also were quantified after digestion with acid buffer (n = 3). (*B*) The serum iron concentrations were determined using atomic absorption spectroscopy (n = 3). (*C*) The iron content in the liver tissues were examined after digestion with acid buffer (n = 3). (*D*, *E*) On the 13th day, the mice were underwent CLP surgery. (*D*) The serum levels of IL-6 were detected 24 h after CLP (n = 5). (*E*) The 7-day survival rate was also assessed (Ad-shHepc1 with low iron treatment, n = 18; Ad-shHepc1 without low iron treatment, n = 19). **P* < 0.05; ***P* < 0.01. CLP = cecal ligation and puncture; IL-6 = interleukin 6; IOD = integral optical density.

functions in tissues and cells after inflammatory and infectious stimuli. Considering that the liver is a major source of hepcidin and a critical organ in sepsis, exploration of the role of hepatic-derived hepcidin in sepsis appears worthwhile. To aid in such exploration, a genetically modified mouse model with liver-specific hepcidin disruption was produced in the current study using hydrodynamic injection of Ad-shHepc1 *via* the tail vein.

Hepcidin functions as a master regulator of iron metabolism, controlling systemic iron trafficking by regulating the transfer of dietary, recycled and stored iron from intracellular compartments to extracellular fluid.[13,15] Hepcidin knockout mice develop systemic iron overload accompanied by iron deficit in the macrophage-rich spleen.[21] Consistent with these findings, the current study showed that hepatic hepcidin knockdown mice had significantly increased serum iron levels both in the steady state and after CLP challenge. Concomitantly, these mice had markedly decreased iron store in the red pulp of the spleen, which may contribute to the higher concentration of serum iron. On the other hand, the increase in serum iron may also be due to increased intestinal iron absorption because of the reduced internalization and degradation of ferroportin in enterocytes of the duodenum in hepcidin knockdown mice. Hepatic hepcidin knockdown also led to a higher iron level in the liver, however, the difference was not significant. Previous study showed that in upstream stimulatory factor 2 knockout mice which are lack of hepcidin expression, iron accumulated in the liver between 60 and 100 days after birth.[46] This suggests that in the present hepcidin knockdown model, it may take longer time to observe significant iron overload in the liver.

Iron is an essential element for all living organisms and has a very particular role in mediating host-pathogen interactions. Host iron status can affect microbial pathogenicity and the outcome of infection by providing the most critical growth-limiting nutrient to potential pathogens or generating toxic free radicals that would cause host tissue damage if not contained.[15,39] Consistent with these findings, in the current study, low hepcidin synthesis resulted in high systemic iron levels, which accelerated the proliferation of the pathogens and increased NADPH oxidase activity, then ultimately led to tissue damage and fatal outcome during polymicrobial sepsis. Knockdown of hepcidin led to low intracellular iron content in the macrophages, impairing the function of macrophages in phagocytosis and bactericidal action possibly *via* affecting cytoskeletal rearrangement and ROS production.[47] This may contribute to the development of sepsis. Interestingly, decreasing the systemic iron level rescued the hepatic hepcidin knockdown mice from lethal sepsis. These findings not only show the important role of iron on sepsis, but also emphasize that the increased hepcidin expression during sepsis is protective and might improve the mortality through decreasing the iron content. Taken together, our data indicates that hepcidin plays important roles in the pathophysiology of polymicrobial sepsis mainly *via* regulating systemic iron status.

However, mice with low hepcidin levels such as hemochromaototic mice ($Hfe^{-/-}$ mice) have been demonstrated to better survival in septicemia or infection with intracellular pathogens such as *Mycobacterium tuberculosis* or *Salmonella typhimurium*.[48,49] This contrasted to the observations in our hepatic hepcidin knockdown model treated with CLP surgery. The discrepancy may be due to the different location of the infecting pathogens and the different approach of iron achieving for the invading microbes. The low iron concentration in macrophages from the hemochromaototic mice ($Hfe^{-/-}$ mice) is unfavorable to the replication of intracellular pathogens.[48,49] However, the high serum iron level in the hepatic hepcidin knockdown mice contributes to the growth of extracellular pathogens such as *Escherichia coli*, *Proteus mirabilis* and *Enterococcus* translocating from enteric microbial flora in CLP-induced polymicrobial sepsis.

Hepcidin may also modulate inflammation.[25,50] In the current study, serum levels of IL-6 at 24 h after CLP were significantly lower in the hepatic hepcidin knockdown mice than in control mice. The mechanism of the effect of hepcidin on cytokine production remains to be elaborated. Previous studies found that knockout of the *Hfe* gene, the protein product of which senses iron status and regulates the expression of hepcidin, attenuated lipopolysaccharides-induced inflammatory responses and reduced TNF-α and IL-6 secretion by macrophages. The decrease in cytokine production was attributed to the presence of a lowered intramacrophage iron level.[51,52] Iron can either regulate the transcriptional level of genes *via* their effects on the transcription factors, or control the posttranscription of certain gene transcripts *via* iron response elements which are present in the untranslated regions of mRNAs and can bind with iron regulatory proteins under conditions of low iron concentration.[52] Although there are no canonical iron response elements in the untranslated regions of the TNF-α or IL-6 mRNAs, iron may exert its effects *via* the AU-rich sequences in the 3′-untranslated regions of TNF-α and IL-6 transcripts, since several proteins can bind these elements and thus impact on both mRNA stability and translation.[53,54] Similarly, the intramacrophage iron level was reduced in the hepcidin knockdown mice in the current study. *In vitro* study has also demonstrated that chelating intracellular iron decreases macrophage TNF-α and IL-6 mRNA levels in response to lipopolysaccharides. There was no significant difference in TNF-α levels observed between the two groups *in vivo* in the current study. Because TNF-α is an early acute inflammatory cytokine, the optimal time for measuring TNF-α concentration might have been missed in the current study. However, despite the lower level of IL-6 in the hepcidin knockdown mice after CLP challenge, the mortality of these mice increased. Since knockdown of hepcidin simultaneously aggravated bacterial infection and oxidase stress *via* enhancing systemic iron level, the advantages of the lower level of pro-inflammatory

cytokines for sepsis might be rather limited in the hepcidin knockdown mice which were iron-overloaded. In contrast, when the systemic iron level was decreased in the hepcidin knockdown mice, repression of hepcidin increase after CLP challenge was protective, which was in accordance with the lower IL-6 level.

Sepsis is a complex disorder that often develops in association with other critical illness such as preexisting liver disease.[55–60] Patients with liver disease have higher risk of infection and sepsis, with a reported incidence of 30–50%.[55] The abnormal susceptibility to infection and sepsis in such patients results from multiple immunologic defects caused by the liver.[55,60] Recent studies have shown that hepatic hepcidin expression is downregulated or impaired in patients with liver diseases such as chronic hepatitis C, alcohol-related liver cirrhosis, and hepatocellular carcinoma.[61–65] The hepatic hepcidin knockdown model used in this study closely mimics hepcidin status under these clinical conditions. Thus, the findings of the current study strongly suggest that hepatic hepcidin is a key factor involved in the pathophysiology of sepsis, especially when accompanied by the complication of liver disease.

Taken together, the results of the current study show that hepatic hepcidin plays an important role in bacterial growth and in the inflammatory response during sepsis through its regulation of host iron status. This conclusion is further supported by the demonstration that decreasing systemic iron levels rescued the hepatic hepcidin knockdown mice from lethal sepsis. These findings not only delineate the pathological mechanisms of hepatic hepcidin in sepsis, but also have potential therapeutic implications for liver diseases in which hepcidin expression is decreased.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Fang: Department of Anesthesiology, The First Affiliated Hospital, School of Medicine, Zhejiang University, QingChun Road 79, 310003 Hangzhou, China. xiangming_fang@163.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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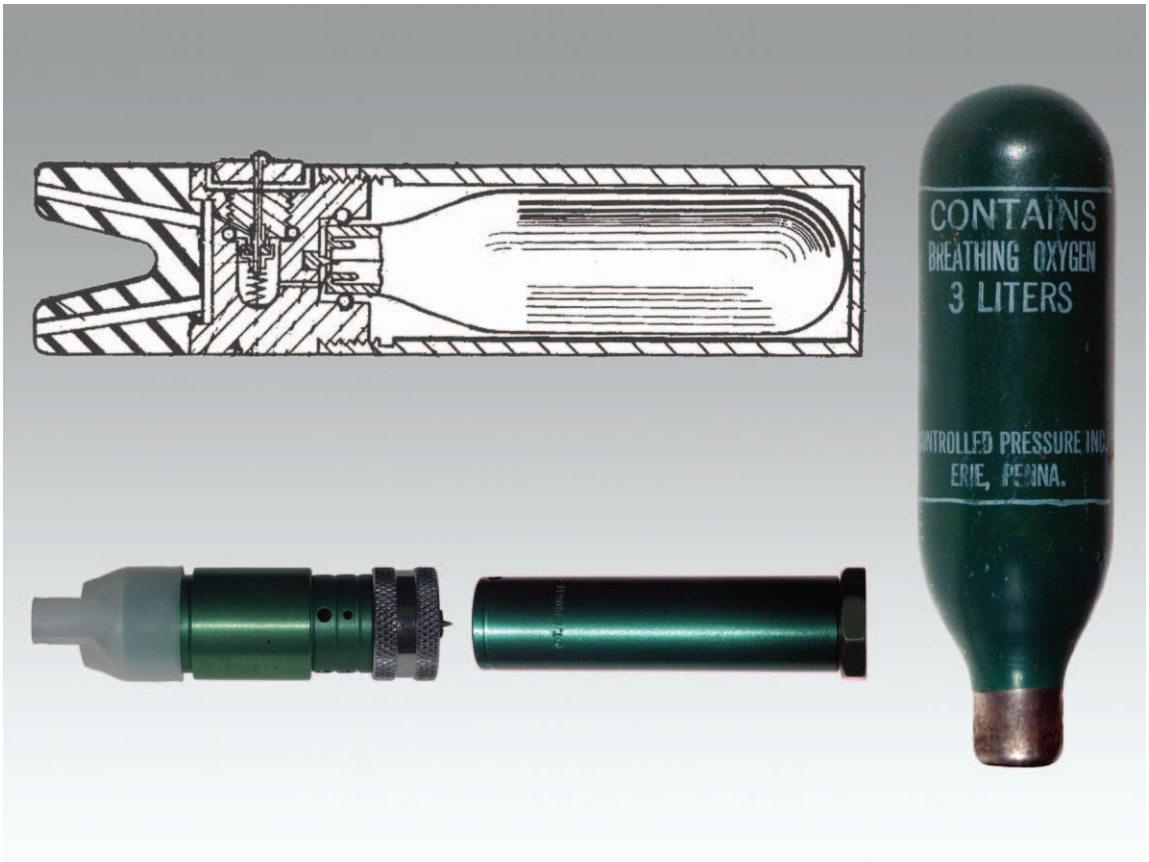
ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

SPAR(klet) WARS: Erie's Duncan *et al. vs.* Cleveland's Hingson *et al.*



"Sparklets" were small compressed gas cartridges that were popularized for medical use by a British physician in in the 1920s. From Erie, Pennsylvania, inventors James Duncan and Leo Trambley filed their U.S. Patent drawing (*high left*) from their "Oxygen dispensing device …" in May of 1955. As distributed by their hometown Controlled Precision, Inc., their "Oxy-Hale" device (*low left*) provided portable or field delivery of 3 liters of oxygen per exchangeable green sparklet (*right*). Advertised nationwide for oxygenating or resuscitating patients, the Oxy-Hale was even featured in the *Anniston Star*, the hometown newspaper of Alabama native, Robert Hingson, M.D. After arriving in Cleveland, Ohio—about 100 miles from Erie, Pennsylvania—Dr. Hingson had collaborated with inventors Frank Ziherl and Arthur Kish to file a patent for their own sparklet device. They filed almost 9 months before the Duncan–Trambley team but would not be granted the more complicated patent until 1960, nearly 20 months after the Erie team. (Copyright © the American Society of Anesthesiologists, Inc.)

George S. Bause, M.D., M.P.H., Honorary Curator, ASA's Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.