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Hepcidin Is Regulated during Blood-Stage Malaria and Plays a Protective Role in Malaria Infection

Hai-Zhen Wang,[1] Ying-Xin He,[1] Chun-Ju Yang, Wei Zhou, and Cheng-Gang Zou

Hepcidin is one of the regulators of iron metabolism. The expression of hepcidin is induced in spleens and livers of mice infected with pathogenic bacteria. Recent studies have indicated that serum hepcidin level is also increased in human subjects infected with *Plasmodium falciparum*. The mechanism of the regulation of hepcidin expression and its role in the infection of malaria remains unknown. In this study, we determined the expression of hepcidin in livers of mice infected with *Plasmodium berghei*. The expression of hepcidin in the liver was upregulated and downregulated during the early and late stages of malaria infection, respectively. Inflammation and erythropoietin, rather than the iron-sensing pathway, are involved in the regulation of hepcidin expression in livers of infected mice. Meanwhile, we investigated the effect of hepcidin on the survival of mice infected with *P. berghei*. Treatment of malaria-infected mice with anti-hepcidin neutralizing Abs promoted the rates of parasitemia and mortality. In contrast, lentiviral vector-mediated overexpression of hepcidin improved the outcome of *P. berghei* infection in mice. Our data demonstrate an important role of hepcidin in modulating the course and outcome of blood-stage malaria. *The Journal of Immunology*, 2011, 187: 6410–6416.

H epcidin, a small peptide synthesized mainly in the liver, is a key regulator of iron homeostasis (1). Hepcidin reduces dietary iron absorption in the small intestine and inhibits iron release from macrophages by a mechanism via promoting the internalization and degradation of ferroportin, the sole known iron exporter (2). Unlike human hepcidin, there are two isoforms of hepcidin (1, 2) in mice (3). The expression of hepcidin is significantly induced by iron overload and inflammation (4). Conversely, hepcidin expression is downregulated in response to low body iron levels, erythropoiesis, and hypoxia (5). In vitro studies have demonstrated that erythropoietin (EPO) inhibits hepcidin expression in hepatocarcinoma cell lines and hepatocytes (6, 7). In addition, upregulation of hepcidin expression is also observed in spleens and livers of mice infected with pathogenic bacteria (8, 9). This response leads to intracellular iron sequestration and reduces the availability of iron, thus inhibiting the growth of pathogens (8, 10).

Malaria is a leading cause of morbidity and mortality, especially in developing countries. Malaria is a complex multisystem disease including erythrocyte invasion and destruction, dysregulated cytokine cascades, and dyserythropoiesis (11). *Plasmodium* spp. parasites produce hemozoin to detoxify the free heme, which is from hemoglobin degradation. After erythrocyte rupture, hemozoin is released into the peripheral blood. Hemozoin is a potent proinflammatory agent in vitro and in vivo (12, 13). Hemozoin has been proposed to activate cytokine production in immune cells through the TLR9 pathway or the IL-1R pathway (14–16). Besides dysregulation of inflammatory responses, profound disturbances in iron homeostasis are frequently observed in individuals with malaria. Iron deficiency is a common condition in many malaria-endemic regions. In addition, malarial anemia leads to hypoxia, which upregulates EPO production in the kidney and serum EPO levels (17). It is possible that these physiological factors might influence the expression of hepcidin during malaria infection.

Recently, several studies have linked the relations between hepcidin and malaria. For instance, Howard et al. (18) have reported that urinary hepcidin is positively associated with parasitemia among patients with acute *Plasmodium falciparum* malaria. Furthermore, urinary concentrations of hepcidin are significantly increased in anemic children with febrile *P. falciparum* malaria or in children with asymptomatic *P. falciparum* and *Plasmodium vivax* parasitemia (19, 20). The mechanism underlying the regulation of hepcidin levels during malaria infection remains unclear. It has been suggested that the elevation of hepcidin concentrations in malaria infection is probably due to a result of suppressed erythropoietic activity (19). The aim of this study was to investigate the putative regulatory pathways of hepcidin expression in vivo during blood-stage infection of *Plasmodium berghei* in mice. We also examined the role of hepcidin in infection of malaria. In this article, we present evidence that hepcidin plays an important role in the protection of mice against malaria infection.

Materials and Methods

Experimental animals and infection

Male ICR and Kunming strain mice, 8–10 wk old, were purchased from the Animal Center, Kunming Medical College (Kunming, Yunnan, China). *P. berghei* ANKA parasites were a kind gift from Dr. Fei Sun (Xinxiang Medical College, Xinxiang, Henan, China). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The protocol of the experiments was approved by the Animal Care and Use Committee of Yunnan University.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BBB, blood–brain barrier; EPO, erythropoietin; TU, transfer units.

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Mice were inoculated i.p. with 10^6 parasitized erythrocytes. Postinfection, a drop of tail-vein blood was collected at the indicated times for parasitemia. Blood smears were stained with Giemsa. Parasitemia was examined by counting at least 1000 cells.

To analyze the levels of hemoglobin and hematocrit, and reticulocyte counts, infected mice were killed at the indicated times. Blood samples were collected and analyzed using the Sysmex XE-2100 hematology analyzer (Sysmex, Kobe, Japan). For the measurement of mRNA levels, a part of the liver was immediately removed and quickly frozen in liquid nitrogen. For immunohistochemical analysis, the remaining part of the liver was fixed in formalin, dehydrated in graded ethylic alcohol, and embedded in paraffin.

Permeability of the blood–brain barrier

When mice infected with *P. berghei* ANKA began showing neurological symptoms, usually on day 6 postinfection, 0.1 ml of 1% (w/v) Evans blue in PBS was injected into the tail vein i.v. Animals were killed 1 h later. Evans blue extravasation was measured by examination of the brain (21).

Assay for cytokines and iron

To analyze the serum levels of cytokines and iron, infected mice were killed at the indicated times. Blood was collected and sera were preserved at −80°C. The serum levels of cytokines were measured by commercially available ELISA kits (R&D Systems, Minneapolis, MN). Serum iron levels were determined using an automated biochemistry analyzer (Hitachi 7060; Hitachi, Tokyo, Japan).

Quantitative real-time RT-PCR analysis

Frozen liver tissues (50 mg) were homogenized in liquid nitrogen. Total RNA was isolated using a total RNA extraction kit (Tiangen Biotech, Beijing, China). Random-primed cDNAs were generated by reverse transcription of total RNA samples with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). A real-time PCR analysis was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Premix-Ex TagTM (Takara, Dalian, China). The primers used for PCR were: hepcidin—forward, 5′-AGA GCT GCA GCC TTT GCA C-3′; reverse, 5′-GAA GAT GCA GAT GGG GAA GT-3′; and β-actin—forward, 5′-AGT GTG ACG TTG ACA TCC GTA-3′; reverse, 5′-GCC AGA GCA GTA ATC TCC TTC T-3′.

Immunohistochemistry

Hydrated, 4.0-μm sections of paraformaldehyde-fixed, paraffin-embedded liver tissues were blocked by 3% BSA-PBS solution at room temperature for 1 h. Then the sections were stained with anti-mouse hepcidin Abs (Santa Cruz Biotech, Santa Cruz, CA) at 4°C overnight. The sections were washed with PBS three times for 10 min at room temperature and incubated with HRP-conjugated goat anti-mouse IgG (Calbiochem, La Jolla, CA) for 60 min at room temperature. The substrate solution diaminobenzidine (Tiangen, Beijing, China) was added until the desired intensity of color developed. Finally, the slides were counterstained with hematoxylin after diaminobenzidine staining.

Western blotting

After liver tissues (25 mg) were homogenized in liquid nitrogen, the homogenate was lysed on ice for 30 min in lysis buffer (containing 0.15 M NaCl, 30 mM Tris, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 1 mM EDTA, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, and 2 mM Na_3VO_4). Sample lysates were resolved by SDS-PAGE (10% gel) and transferred to polyvinylidene difluoride membranes. Primary Abs were anti-STAT3, anti-SMAD1/5/8 Abs (Santa Cruz Biotech), anti-phospho (Tyr705) STAT3 Abs (Epitomics, Burlingame, CA), anti-phospho (Ser463/465) SMAD1/5/8 Abs (Millipore, Temecula, CA), and anti-actin Abs (Sigma, St. Louis, MO). The secondary Abs were a peroxidase-coupled anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ). The membrane was exposed to ECL Hyperfilm (Amersham Biosciences), and the film was developed.

Preparation of synthetic hemozoin

Hemozoin was synthesized according to the method described previously (12). In brief, 45 mg hemin chloride (Sigma) was solubilized in 4.5 ml of 1 N NaOH and then treated with 450 μl of 1 N HCl. After 10.2 ml of 1 M sodium acetate (pH 4.8) was added, the suspension was stirred for 2–3 h at 60°C. After addition of 150 μl 10% SDS, the suspension was centrifuged for 15 min at 14,000 × g. The pellet was sonicated at lowest power setting in 100 mM sodium bicarbonate (pH 9.0) in 0.5% SDS, and again centrifuged. The pellet was washed four times in 2% SDS and then in water to remove SDS. The hemozoin was dried at 37°C overnight, then

resuspended in endotoxin-free PBS (Life Technologies, Rockville, MD) at a final concentration of 2.5 mg/ml. Absence of contamination by endotoxins was confirmed as measured by the *Limulus* amebocyte lysate test using E-toxate kit (Sigma). The hemozoin was sonicated in a bath sonicator for 1–2 min before use.

Drug injection

Chloroquine and *Salmonella abortus equi* LPS were purchased from Sigma. Chloroquine (25 mg/kg) was injected i.p. once daily, on days 0, 1, 2, and 3 postinfection in ICR mice, using PBS as a vehicle. LPS (1 mg/kg) was injected i.p. once daily, on days 0, 1, 2, and 3 postinfection in ICR mice, using olive oil with dimethyl sulfoxide (96:4) as a vehicle (22). ICR mice were injected i.v. into the tail vein with hemozoin (100 μg per mouse; suspension of insoluble material).

Treatment of anti-hepcidin and anti–IL-6 Abs

Anti-mouse hepcidin Abs (1 mg/kg; Santa Cruz Biotech) or isotype control Abs (mouse IgG 1 mg/kg; Amersham) were injected i.v. into the tail vein of ICR mice at the same time when *P. berghei* ANKA was inoculated. Injection of the Abs was repeated for the next 3 d. Parasitemia was monitored at designated times. For Kunming mice, anti-human hepcidin mouse mAbs (1 mg/kg; Santa Cruz Biotech) were tested.

Anti-mouse IL-6 Abs (1 mg/kg; Santa Cruz) or an isotype control Ab (mouse IgG 1 mg/kg; Amersham) was injected i.v. into the tail vein of ICR on days 2–4 postinfection. The expression of hepcidin was determined at designated times.

Construction of the lentiviral vectors, lentivirus production, and purification

Lentivirus vector expressing mouse hepcidin-1 and lentivirus particles was obtained by Shanghai R&S Biotechnology (Shanghai, China). In brief, mouse hepcidin-1 cDNA amplified from liver-derived cDNA samples was inserted into BamHI and NheI restriction enzyme sites within the multiple cloning site of the lentivirus vector pLenti6.3-IRES2-EGFP/V5 DEST (pLenti-EGFP), resulting in the vector pLenti-Hep-EGFP.

The lentivirus vector pLenti-EGFP or pLenti-Hep-EGFP was cotransfected with three other helper plasmids (pLP1-Gag-pol, pLP-VSVG, and pLP2-Rev) into human embryonic kidney 293T cells. After 24-h transfection, the EGFP fluorescence could be seen under a fluorescence microscope. The supernatant was collected 48 h after transfection, and fresh medium was added to the culture flask. After the cells were cultured for another 24 h, the supernatant was collected again. The virus vector-containing supernatants collected from 48 and 72 h were mixed. The mixture was centrifuged at 3000 rpm × 15 min at 4°C. Then the liquid was filtered by a 0.45-μm filter membrane. The acquired virus was stored at −80°C until use. The titers of the pLenti-Hep-EGFP and pLenti-EGFP vectors were 1–3 × 10^9 transfer units (TU/ml).

Animal infection with lentiviruses

Viral supernatants were injected i.v. via the tail vein of ICR mice. Each mice received 1 × 10^8 TU of the respective infectious viral particles. The mice were allowed to recover in a warm cage and sacrificed at days 3, 7, and 14 after the injection to determine the levels of hepcidin mRNA.

Statistical analysis

Data from experiments were expressed as mean ± SD. Statistical difference between the groups was analyzed using one-way ANOVA followed by a least significant difference test. Pearson correlation coefficient analysis was performed to determine the association between hepcidin and one of the variables. The hypothesis that two variables are uncorrelated was tested. The log-rank test was used to analyze the statistical difference in the survival rates among different treatment groups. A *p* value <0.05 was considered to be significant. Statistical analyses were performed by use of SPSS11.1 software.

Results

Hepcidin expression in liver after infection with P. berghei

After infection with *P. berghei* ANKA, ICR mice showed severe symptoms of malaria. Parasitemia significantly increased on day 4 and peaked on day 5 postinfection (Fig. 1A). During infection, mice gradually developed anemia. There was obvious loss of erythrocytes through parasite maturation. The hematocrit levels began to decrease on day 5 postinfection and declined to the lowest level on day 8 postinfection (Fig. 1B).
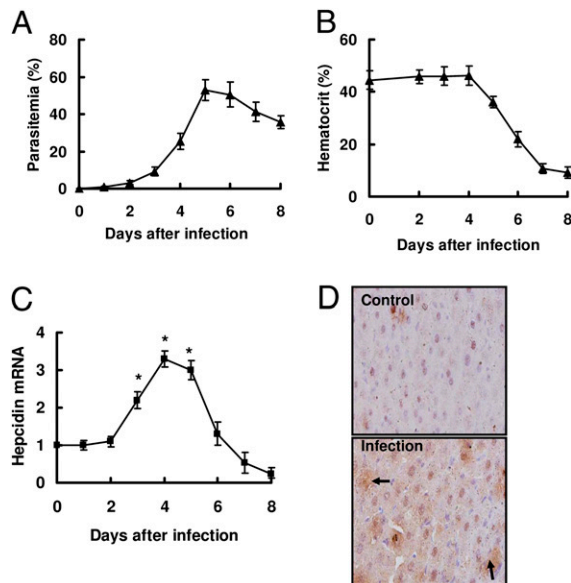
FIGURE 1. Time course of hepatic hepcidin expression in ICR mice during malaria infection. ICR mice were infected with *P. berghei*. *A*, Parasitemia are shown ($n = 5$ for each group). *B*, Hematocrit levels are shown ($n = 5$ for each group). *C*, Total RNA was extracted from liver tissue and subjected to real-time PCR. All results are standardized to the levels of β-actin. These results are means ± SD ($n = 5$ for each group). *$p < 0.05$ versus control. *D*, The protein expression of hepcidin in the liver was determined by immunohistochemical staining on day 4 postinfection. Representative examples were shown in control mice and infected mice, respectively. Arrows indicate positive hepatocytes (original magnification ×400). Similar results were obtained in three independent experiments.

A major feature of cerebral malaria pathology after infection with *P. berghei* ANKA is the alteration of the blood–brain barrier (BBB) (23). To assess the development of cerebral malaria during infection, we infected ICR mice with *P. berghei* ANKA and the BBB integrity was determined by an Evans blue dye exclusion assay. As shown in Supplemental Fig. 1*A*, the brains of infected mice were stained by Evans blue administered i.v. on day 6 postinfection, indicating a disruption of the BBB integrity.

The expression of hepatic hepcidin was determined using real-time PCR during infection with *P. berghei*. As shown in Fig. 1*C*, the mRNA levels of hepcidin significantly increased on day 3 of infection and attained a peak on day 4 postinfection. The expression of hepatic hepcidin declined to the baseline level on day 6 postinfection and attained the lowest level, which was only 23% of the control value, on day 8 postinfection. In addition, immunohistochemistry analysis revealed a significant increase in hepcidin protein levels in the liver on day 4 of infection (Fig. 1*D*).

Iron status during infection

Hepcidin levels are upregulated by iron, which requires signaling through the bone morphogenetic protein and Sma- and Mad-related protein 4 (BMP/Smad4) pathways (24–26). We thus examined the time course of these regulators in mice after infection with *P. berghei*. As shown in Fig. 2*A*, the serum iron levels did not change during 2 d of infection and significantly decreased on days 3–5 postinfection. The serum iron significantly increased on days 7 and 8 postinfection. To ascertain the potential role of this signaling pathway in the regulation of hepcidin by iron, we examined the activation of Smad1/5/8 in livers of mice infected with malaria. As shown in Fig. 2*B*, the phosphorylation of Smad1/5/8 did not significantly change in the liver during infection with *P. berghei*.
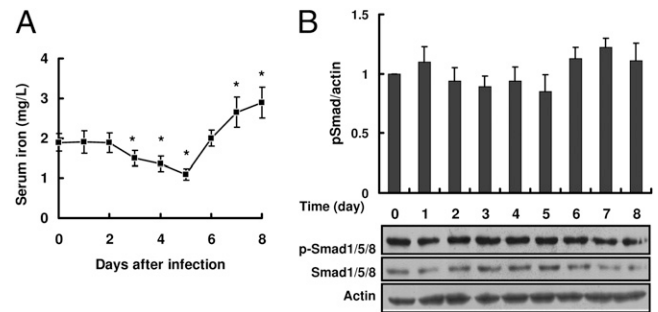

FIGURE 2. The expression of hepcidin is not associated with iron status during infection. ICR mice were infected with *P. berghei*. *A*, Time course of serum iron levels. These results are means ± SD ($n = 5$ for each group). *$p < 0.05$ versus control. Similar results were obtained in three independent experiments. *B*, The protein levels in the liver were detected by Western blotting. The *upper panel* shows quantification of immunoreactivity levels. Data are expressed as percentage change from control and are representative of data obtained in three similar experiments.

These results indicate that iron-mediated, BMP-responsive Smad activation is not involved in the regulation of hepcidin expression.

Levels of inflammatory factors and EPO during infection

The expression of hepcidin is regulated by inflammation through macrophage production of proinflammatory cytokines, such as IL-6 (27, 28). We thus determined the serum levels of proinflammatory cytokines by ELISA. Mice showed a 20-fold increase in IL-6 levels (Fig. 3*A*) and a 4-fold increase in TNF-α levels (Fig. 3*B*) on day 3 postinfection. TNF-α levels returned to the basal level on day 5 postinfection. IL-6 levels declined but were still higher than the baseline levels on days 7–8 postinfection. Because EPO downregulates the expression of hepcidin, we also determined the levels of serum EPO after infection with *P. berghei*. There was a significant increase in EPO production after the development of malarial anemia on day 6 postinfection. The levels of serum EPO continued to increase on days 7–8 postinfection when these mice were most anemic.

IL-6 induces hepcidin expression by the activation of STAT3 in hepatocytes (29). Because the serum levels of the proinflammatory cytokine were dramatically increased, we determined the phosphorylation of STAT3 in the liver postinfection. We found that the phosphorylation of STAT3 was markedly increased on days 3–5 postinfection and declined on days 6–8 postinfection (Fig. 3*D*). To further confirm the role of IL-6 on the expression of hepcidin, ICR mice received an i.v. injection of anti–IL-6 neutralizing Abs or an equal dose of mouse secondary anti-IgG Abs at 1 mg/kg daily on days 2–4 postinfection. As shown in Fig. 3*E*, the induction of hepcidin expression by malaria infection was significantly inhibited after the treatment of anti–IL-6 neutralizing Abs.

Correlation analysis among hepcidin mRNA levels and selected laboratory parameters

Correlations between selected laboratory parameters and hepcidin mRNA levels were calculated. We found that there was a positive correlation of hepcidin mRNA levels with concentrations of the cytokines IL-6 and TNF-α ($p < 0.001$). Hepcidin mRNA levels were inversely associated with serum levels of iron and EPO ($p < 0.001$).

Injection of hemozoin increases IL-6 levels and hepcidin expression

Previous studies have identified that hemozoin stimulates the production of IL-6 through the TLR9 pathway or the IL-1R pathway (12, 14–16). In this study, hemozoin-containing monocytes in the

FIGURE 3. Time course of inflammatory factors and EPO during infection. ICR mice were infected with *P. berghei*. Time course of serum IL-6 (*A*), TNF-α (*B*), and EPO levels (*C*). These results are means ± SD (*n* = 5 for each group). *$p < 0.05$ versus control. Similar results were obtained in three independent experiments. *D*, The protein levels in the liver were detected by Western blotting. The *upper panel* shows quantification of immunoreactivity levels. Data are expressed as percentage change from control and are representative of data obtained in three similar experiments. *$p < 0.05$ versus control. *E*, Anti-mouse IL-6 Abs (infection + aIL-6) or an isotype control Ab (infection) was injected i.v. into the tail vein of ICR on days 2–4 postinfection. The mRNA levels of hepcidin in the liver were determined on day 5 postinfection. These results are means ± SD (*n* = 5 for each group). Similar results were obtained in three independent experiments. *$p < 0.05$ versus infection group.
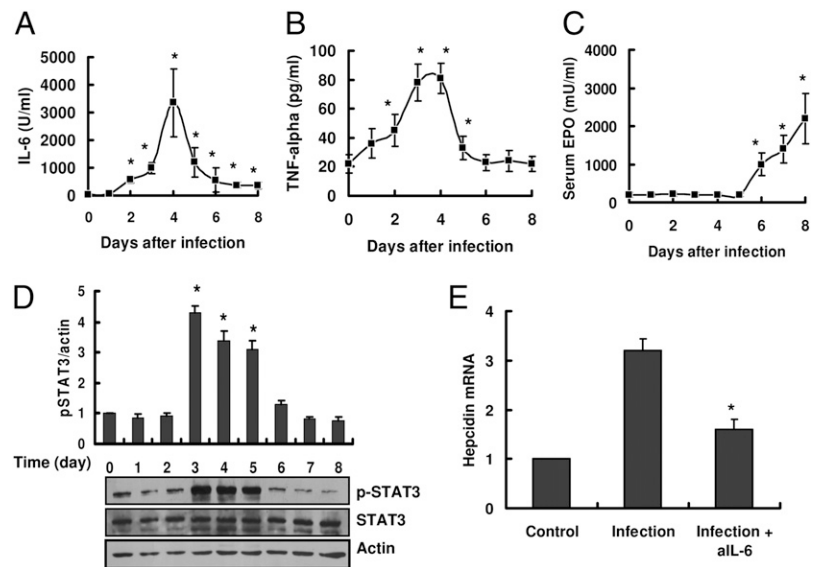
peripheral circulation were observed on day 3 postinfection (Supplemental Fig. 1*B*). The injection of synthetic hemozoin resulted in a significant increase in the expression of hepcidin in the liver and the levels of serum IL-6 (Fig. 4).

Neutralization of hepcidin in mice

A previous study has demonstrated that pretreatment of LPS (4 mg/kg) for 3 d increased survival in mice inoculated with lethal *P. berghei* (22). We found that ICR mice treated with a sublethal dose of LPS (1 mg/kg) once a day for 4 consecutive days had significantly lower parasitemias postinfection (Fig. 5*A*). As expected, control animals developed fulminant infection, and all died on day 11 postinfection. However, 6 of 15 mice from the groups receiving LPS survived infection (Fig. 5*B*). In a parallel experiment, all mice treated by chloroquine survived and cleared their blood parasitemia by day 10 of infection (data not shown). Previous studies indicate that hepatic hepcidin expression is up-

regulated in mice after injection of LPS (4, 30). LPS also induced an increase in urine hepcidin levels after injection in human volunteers (31). In this study, LPS induced an increase in hepatic expression in ICR mice at 4-h administration and kept the high levels at 8 h (Fig. 5*C*).

Based on the data, we hypothesized that the protective effect of LPS against malaria is probably due to the induction of hepcidin expression. ICR mice received an i.v. injection of anti-hepcidin neutralizing Abs at 1 mg/kg daily on days 1–3 postinfection, to investigate whether hepcidin plays a role in protecting mice against malaria. Treatment with anti-hepcidin Abs significantly exacerbated the outcome of malaria. The rates of parasitemia and reticulocytemia in anti-hepcidin Ab–treated mice were significantly higher than those in IgG-treated control mice (Fig. 5*D*, Supplemental Fig. 2*A*). In contrast, the hematocrit levels were significantly lower in anti-hepcidin–treated mice than in control mice (Supplemental Fig. 2*B*). One hundred percent of anti-hepcidin–treated mice died on day 8 postinfection compared with 60% of control mice (Fig. 5*E*).

We also tested the effect of anti-hepcidin neutralizing Abs on the survival rate of malaria-resistant Kunming mice. Some of Kunming strain mice could survive postinfection. After injection of anti-human hepcidin neutralizing Abs, the rates of parasitemia in anti-hepcidin–treated mice were significantly higher than those in IgG-treated control mice (15 versus 2% on day 4; 45 versus 14% on day 6 postinfection; Supplemental Fig. 3*A*). One hundred percent of anti-hepcidin–treated mice died postinfection compared with 67.7% of control mice (Supplemental Fig. 3*B*).

Gene delivery of mouse hepcidin improves the outcome of malaria infection in mice

To establish the causal relationship between augmented endogenous hepcidin levels and alleviation of malarial anemia, hepcidin was overexpressed via lentiviral vector-mediated gene transfer. Injection of lentiviral vector into ICR mice via the tail vein can result in infection of livers and spleens in mice (32, 33). The mice were sacrificed 3, 7, and 14 d after lentiviral gene delivery. As shown in Fig. 6*A* and 6*B*, the mRNA and protein levels of hepcidin in mice 7 d after pLenti-Hep-EGFP injection were markedly higher than those in mice receiving pLenti-EGFP, respectively. The levels of hepcidin mRNA persisted for at least 14 d. Upon *P. berghei* challenge, the parasitemia progressed to a high level in animals receiving pLenti-EGFP (Fig. 6*C*). All the mice died by
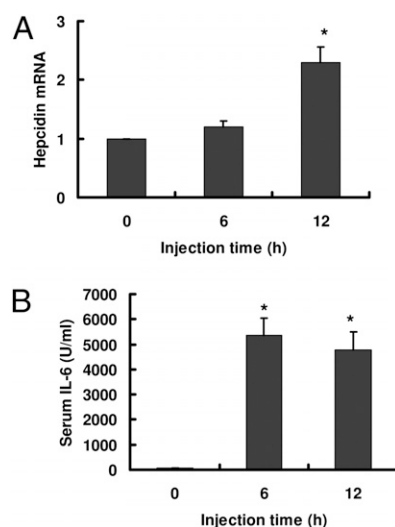
FIGURE 4. IL-6 levels and hepcidin expression are elevated after hemozoin injection. ICR mice were infected with hemozoin (100 μg). *A*, Total RNA was extracted from liver tissue and subjected to real-time PCR. All results are standardized to the levels of β-actin. *B*, The serum IL-6 levels were determined by ELISA. These results are means ± SD (*n* = 5 for each group). Similar results were obtained in three independent experiments. *$p < 0.05$ versus control.

FIGURE 5. Alteration of hepcidin levels affects the rates of parasitemia and survival in mice. *A–C*, ICR mice were injected with LPS (1 mg/kg) once a day for four consecutive days after infection with *P. berghei*. Parasitemia (*A*) and survival rates (*B*) are shown (*n* = 15 for each group). *p* < 0.05 versus control. *C*, After ICR mice were injected with LPS (1 mg/kg), total RNA was extracted from liver tissue and subjected to real-time PCR. All results are standardized to the levels of β-actin. *p < 0.05 versus control. *D* and *E*, ICR mice were treated daily with anti-mouse hepcidin neutralizing Abs or control IgG once a day for four consecutive days after infection with *P. berghei* (*n* = 10 for each group). Parasitemia (*D*) and survival rates (*E*) were determined. Similar results were obtained in three independent experiments. *p < 0.05 versus control.
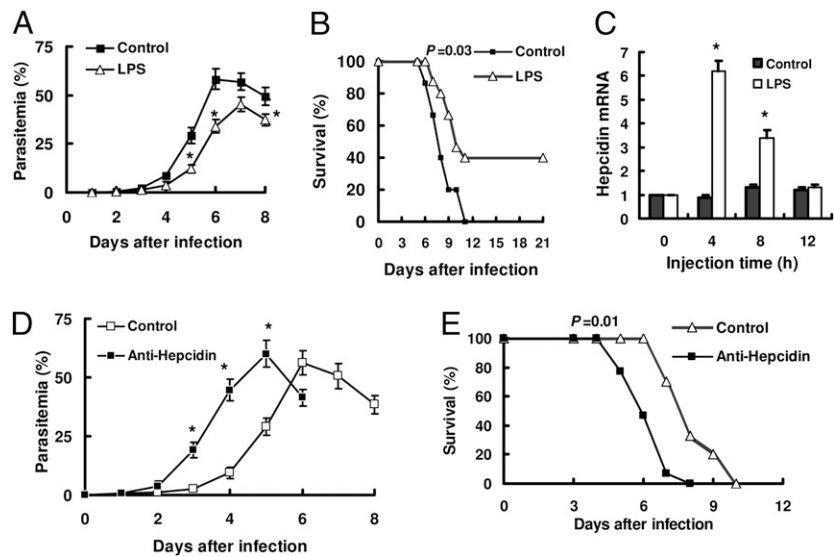
day 12 postinfection (Fig. 6D). In contrast, Hep-overexpressing animals exhibited a marked reduction in the levels of parasitemia (Fig. 6C), the hematocrit levels (Supplemental Fig. 4A), and the leakage of Evans blue dye (Supplemental Fig. 1). The reticulocytemia levels in Hep-overexpressing mice were lower than those in control mice (Supplemental Fig. 4B). Sixty percent of Hep-expressing mice survived (Fig. 6D). These mice subsequently developed protective immunity against *P. berghei*, as demonstrated by their survival 3 wk after primary infection.

Discussion

Hepcidin represents a central regulator of iron homeostasis. Upregulation of hepcidin expression has been observed in livers of mice infected with pathogenic bacteria (8, 9). Recent studies have indicated that urinary or serum hepcidin levels are increased in patients with malaria (18–20, 34). However, little is known about the regulation of hepatic hepcidin expression during malaria infection. In this study, results from our murine model have demonstrated that the expression of hepcidin in the liver is upregulated and downregulated during the early and late stages of malaria infection, respectively. Another main finding of this study is that

hepcidin plays a protective role in the defense against the infection of malaria.

IL-6 is one of the most potent inflammation factors in promoting the expression of hepcidin by the activation of STAT3 in the liver (27, 28). In this study, STAT3 phosphorylation in livers of ICR mice was increased after an increase in the levels of IL-6 after malaria infection. In addition, correlation analysis revealed that hepatic hepcidin expression positively correlated with levels of serum IL-6 in mice. After submission of this manuscript, Portugal et al. (35) reported that the expression of hepcidin was upregulated 5-fold in livers of mice with an ongoing blood-stage infection after injection of mice with *P. berghei* NK65 sporozoite. These authors also showed that hepcidin upregulation in primary hepatocytes induced by sera of blood-stage infected mice was markedly suppressed by IL-6–specific Abs. We also found that the induction of hepcidin expression in livers of mice with *P. berghei* infection was significantly inhibited after the treatment of anti–IL-6 Abs (Fig. 3E). Based on these results, we suggest that IL-6 is responsible for the induction of hepcidin expression in the liver after malaria infection. Previous studies have identified that hemozoin stimulates the production of IL-6 through the TLR9 pathway or the
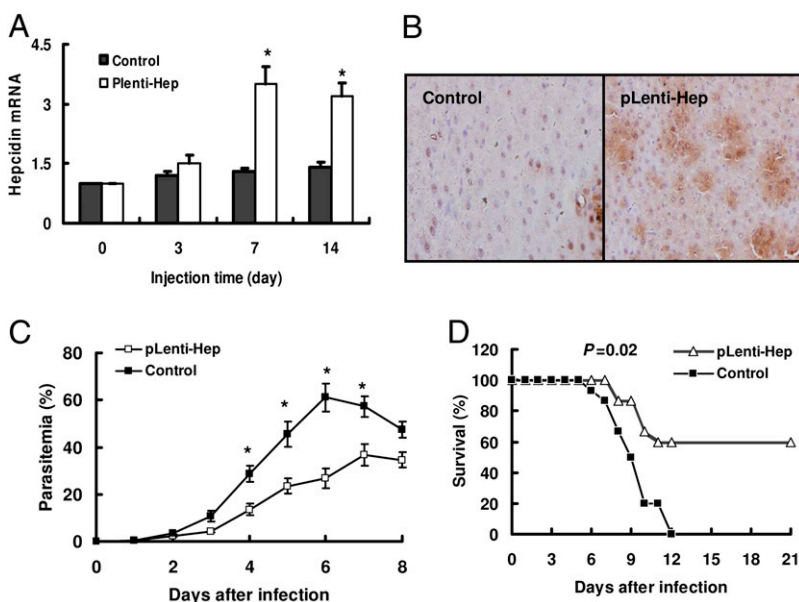

FIGURE 6. Effect of hepcidin overexpression after lentiviral gene delivery on the rates of parasitemia and survival in mice. *A* and *B*, pLenti-Hep-EGFP injection (10^8 TU/mouse) was injected via the tail vein, and the animals were euthanized after 3, 7, or 14 d. *A*, The levels of hepcidin mRNA were measured (*n* = 4 for each group). *p < 0.05 versus control (pLenti-EGFP). *B*, The protein expression of hepcidin in the liver was determined by immunohistochemical staining on day 7 after i.v. injection. Representative examples were shown in control mice and mice with pLenti-Hep-EGFP, respectively (original magnification ×400). *C* and *D*, After ICR mice were treated with pLenti-Hep-EGFP via i.v. injection for 7 d, the mice were infected with *P. berghei*. Parasitemia (*C*) and survival rates (*D*) were determined (*n* = 10 for each group). Similar results were obtained in three independent experiments. *p < 0.05 versus control.

IL-1R pathway (12, 14–16). In this study, hemozoin was observed in monocytes on day 3 postinfection. The injection of synthetic hemozoin promoted the levels of serum IL-6 and induced the expression of hepcidin in the liver, suggesting that it is one of the major components released by *Plasmodium* spp. that is responsible for the induction of hepcidin expression during the early stage of infection.

The expression of hepcidin in the liver was downregulated on days 7 and 8 postinfection. At the same time, ICR mice had the lowest levels of hematocrit, thus suffering from severe anemia. When anemia occurs, EPO production is significantly stimulated by the kidney (36). In this study, the levels of serum EPO were significantly increased, which was accompanied with a decrease in the expression of hepcidin on days 6–8 postinfection. A negative correlation was found between the levels of hepcidin mRNA and serum EPO. A recent study has demonstrated that EPO inhibits the induction of IL-6 in macrophages after LPS treatment or in spleens of mice after *Salmonella* infection (37). The action mediated by EPO is attributable to blockage of NF-κB p65 activation. Thus, the reduction of serum IL-6 levels was probably due to an increase in EPO levels on days 6–8 postinfection. It has been shown that EPO significantly inhibits the expression of hepcidin in livers of mice induced by LPS via suppression of the STAT3 signaling pathway (38). Indeed, a decrease in the phosphorylation of STAT3 was observed on days 6–8 after malaria infection. Thus, the increase of EPO is responsible for reducing the expression of hepcidin, probably by inhibiting the production of IL-6 and subsequent STAT3 phosphorylation in the liver.

The iron-sensing pathway that upregulates the expression of hepcidin involves the activation of BMP/Smad4 signaling, leading to the generation of phosphorylated R-Smads. However, our results suggest that the iron-sensing pathway is unlikely to be involved in the upregulation of hepcidin expression. Whereas the levels of serum iron significantly decreased, the expression of hepatic hepcidin markedly increased on days 3–5 postinfection. Furthermore, the infection of malaria did not result in the activation of the Smad4 pathway, as judged by the induction of Smad1/5/8 phosphorylation. However, correlation analysis of our data reveals an inverse relationship between the levels of iron and hepcidin expression. This observation is consistent with a recent study of de Mast and colleagues (34). The results from these authors have indicated that an increase in serum hepcidin levels is associated with a decline in serum iron concentrations in human volunteers infected with *P. falciparum*. Thus, the reduction of serum iron during the early stage of infection is probably due to the upregulation of hepcidin.

The expression of hepcidin is upregulated during infection of pathogens, suggesting that hepcidin plays an important role in the host defense, probably by limiting the availability of iron after invading of microorganisms (8, 10). Recent studies have indicated that the increased urinary hepcidin levels were associated with iron maldistribution, as was indicated by observations of hypoferremia and high serum concentrations of ferritin. Antimalarial treatment resulted in a rapid decrease in urinary concentrations of hepcidin and reversal of the hypoferremia (18, 19). Our study demonstrated that increased hepcidin at the early stage of infection plays a protective role in the defense against the infection of malaria. We found that treatment of mice with anti-hepcidin Abs resulted in an increase in parasitemia and mortality. In contrast, gene delivery of hepcidin improved the outcome of *P. berghei* infection in mice. Because hepcidin downregulates the levels of serum iron, these results suggest that the limitation of iron availability confers a benefit to the host against malaria. In agreement with our results, Portugal et al. (35) have also demonstrated that increased hepcidin inhibits *Plasmodium* liver infection in mice by reducing iron availability. Indeed, parasitemia was significantly decreased and survival was significantly enhanced in iron-deficient mice after infection with *P. berghei* (39). Iron overload in BALB/c mice promotes the hepatic development of *Plasmodium yoelii* in vivo and in vitro (40). Taken together, these results suggest that hosts develop an iron-withholding defense system by induction of hepcidin against malaria infection during the early stage of infection.

Mice that received a sublethal dose of LPS showed an increased survival rate. Previous studies have shown that the antiplasmodial mechanism of LPS or cytokines such as TNF-α appears to be associated with the formation of NO (41, 42). However, we suggest that LPS exhibits the protective effect against malaria infection, at least partly, by inducing hepcidin expression. Human hepcidin exhibits both weak fungicidal activity and cytotoxicity at a concentration (10 μM) that is ~2000-fold higher than that found in human blood (1, 43). Thus, it seems unlikely that hepcidin directly inhibits the growth of malarial parasite.

In conclusion, our study shows that inflammation and EPO, rather than the iron-sensing pathway, are involved in the regulation of hepcidin expression in the liver after malaria infection. The increased levels of hepcidin mRNA result in a decrease in iron serum levels during the early stage of malaria infection. The activation of the iron-withholding system mediated by hepcidin is a probable strategy for hosts against malaria infection.

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Disclosures

The authors have no financial conflicts of interest.

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