**Inappropriate expression of hepcidin by liver congestion contributes to anemia and relative iron deficiency**

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Short title: Anemia in heart failure with liver congestion

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Abbreviations: BA: bleeding anemia; BMP: bone morphogenetic protein; CRAIDS: cardiorenal–iron deficiency syndrome; HA: hemolytic anemia; Hb: hemoglobin; HF: heart failure; KO: knock-out; LC: liver congestion; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; PHL: phlebotomy; PHZ: phenylhydrazine; RID: relative iron deficiency; SEM-EPMA: scanning electron microscopy and electron probe microanalysis; TIBC: total iron-binding capacity; TLR: Toll-like receptor; TR: tricuspid regurgitation; UIBC: unsaturated iron-binding capacity; WT: wild-type

**Abstract**

**Background:** Anemia and relative iron deficiency (RID) are prevalent in patients with heart failure (HF). The etiology of anemia and RID in HF patients is unclear. Hepcidin expression may be closely related to anemia and RID in HF patients. Although hepcidin is produced mainly by the liver and the most frequent histologic appearance of liver in HF patients is congestion, the influence of liver congestion (LC) on hepcidin production has not yet been investigated. We investigated whether hepcidin contributed to anemia and RID in rats with LC.

**Methods and Results:** LC was induced in rats by ligating the inferior vena cava and compared with bleeding anemia (BA) model by phlebotomy and hemolytic anemia (HA) model induced by injection of phenylhydrazine. BA and HA strongly suppressed expression of hepcidin in liver, and so did not cause decrease in serum iron and transferrin saturation. However, hepcidin expression did not decrease in LC rats, which resulted in anemia and lower transferrin saturation. In addition, many cells with hemosiderin deposits were observed in the liver and spleen and not in the bone marrow, and this appeared to be related to suppression of hepcidin expression. Iron accumulated in hepatocytes and bone morphogenetic protein 6, which induces hepcidin, increased. Inflammation was observed in the congestive liver and there was an increase in IL-6, which also induced hepcidin and was induced by free heme and hemoglobin via Toll-like receptor 4.

**Conclusions:** We conclude that LC contributes to RID and anemia, and does so via inappropriate expression of hepcidin.

## Introduction

Anemia frequently occurs in patients with heart failure (HF) and is an independent prognostic factor for mortality.[1] The etiology of anemia in HF patients is multifactorial.[2] In most of the cases, more than one mechanism is often involved. The important factors are hemodilution,[3-5] chronic inflammation,[6] renal dysfunction,[7] hemolysis,[8,9] gastrointestinal bleeding,[10] bone marrow dysfunction,[11,12] resistance to erythropoietin,[13] and hematinic deficiencies including vitamin B12, folic acid, and relative iron deficiency (RID). [14,15] In particular, RID has been proposed to be the most common cause of anemia in patients with advanced HF.[16-19] and a new syndrome, namely, cardiorenal-anemia-iron deficiency syndrome (CRAIDS) has been proposed.[20] In this connection, a study of iron therapy for anemia in patients with HF was reported and another is currently under way.[21,22]

Hepcidin is known to be a main regulator of iron metabolism[23] and this has raised the possibility that hepcidin is closely related to mechanism of anemia and RID in HF patients. Hepcidin regulates intestinal iron absorption and iron recycling by macrophages[24] and hepcidin overproduction leads to a hypoferremic state that impairs the delivery of iron to maturing erythroid cells in the BM.[25,26] Hepcidin is transcriptionally regulated by several factors.[27] Anemia and hypoxia strongly regulate hepcidin, and holotransferrin[28] and intracellular iron concentrations induce hepcidin transcription via bone morphogenetic protein (BMP)-6.[29,30] Hepcidin gene transcription is also responsive to stimulation by IL-6.[30]

However, the relationship between hepcidin and anemia/RID in HF patients has not been fully elucidated and remains controversial.[31,32] Hepcidin is mainly produced by the liver.[33,34] Therefore, anemia/RID in HF patients are thought to be influenced by the level of hepcidin production in the liver. Because the most frequent histopathological finding in the liver of HF patients is congestion,[35] the relationship between hepcidin production and liver congestion (LC) is an important topic to be elucidated; however, this relationship has not been investigated.

Previously we reported that a patient with severe tricuspid regurgitation (TR) with refractory anemia and a very high serum hepcidin level recovered from anemia after valve replacement, which was accompanied by a decrease in the hepcidin level and an improvement of LC.[36] This case suggested that LC may induce the expression of hepcidin and result in exacerbated anemia. In addition, free heme and hemoglobin (Hb) released from destruction of intraparenchymal red blood cells are reported to have several effect such as cytotoxic, excitotoxic, oxidative, and inflammatory effects.[37] We also reported that free heme strongly induced various pro-inflammatory proteins[38] and free heme is known to be key regulator of major mammalian cellular functions.[39] Moreover, free heme and Hb have been reported to have inflammatory effects in tissues with hemorrhage via Toll-like receptor (TLR)4.[40,41]

In this study, we examined the relationship of hepcidin production and anemia/RID in LC. LC was induced in rats by ligating the inferior vena cava (IVC) and these rats were compared with rats in which anemia was induced by

phlebotomy (PHL), i.e., bleeding anemia (BA) model and rats in which hemolytic anemia (HA) was induced by injecting phenylhydrazine (PHZ). In addition, we examined expression of BMP-6 and IL-6, which are hepcidin inducers, and the effect of Hb released within the extracellular space of the congestive liver via TLR4.

## Methods

A complete Methods section is presented in the Supplementary material online, Methods

### Animal model

Lewis rats were obtained from Charles River, and were maintained in our animal facilities until they reached 8 weeks of age for use in creating anemia models. TLR4 knock-out (KO) mice were obtained from Oriental Bioservice Inc. TLR4KO mice and wild-type (WT) littermate mice, aged 8–10 weeks, and with a C57BL/6 background from Charles River, were used in the study. All animal experiments followed the guidelines for the care and use of laboratory animals published by the US National Institutes of Health. All animals were euthanized by inhalation of isoflurane in a euthanasia chamber. Death of the animals was confirmed by monitoring the absence of breath after removal of the carcase from the chamber.

### Rat models of liver congestion

All rats were anesthetized with an intraperitoneal injection of anesthetic agents. LC was induced in rats by ligating the abdominal portion of the IVC as previously reported.[42] Sham-operated control rats underwent the same operation without ligation. The animals were killed on day 4, day 7, day 28, day 42, and day 84 after the operation. Liver, spleen and bone marrow from femurs were collected for gene expression and histological analysis.

**Rat models of bleeding anemia and hemolytic anemia**

Six rats were bled by PHL as BA rats. The BA rats were killed on day 2 and day 14 after first PHL. Nine rats received intraperitoneal injection of PHZ for 2 days as HA rats. The HA rats were killed day 2, day 4, and day 14 after the first injection. Liver, spleen, and bone marrow from femurs were collected for gene expression analysis and histological analysis.

**Blood count and serum iron measurement**

Red blood cell count, blood Hb concentration, and hematocrit, were determined using the automated XE-2100 analyzer and serum iron and unsaturated iron-binding capacity (UIBC) were determined using an automated system H7700.

**Serum hepcidin and IL-6 measurement**

Serum hepcidin and IL-6 concentrations were determined by an Enzyme-linked Immunosorbent Assay Kit for rat hepcidin (Uscn Life Science Inc.,

Wuhan, China) and IL-6 (R&D Systems Inc, Minneapolis, MN, USA).

**Histology and immunohistochemistry**

Specimens from tissue samples were reacted with mouse anti-CD68 antibody and mouse anti-MHC Class II antibody and immunodetection was performed using biotinylated anti-rabbit and anti-mouse immunoglobulins followed by horseradish peroxidase-conjugated streptavidin and 3,3-diaminobenzidine substrate. Each section was also stained with haematoxylin–eosin stain and Berlin blue stain for iron.

**Electron probe microanalyzer**

The chemical composition and ultrastructure of the prepared samples were studied using scanning electron microscopy and electron probe microanalysis (SEM-EPMA). The distribution of amino nitrogen in the pathologic image was also mapped for each sample.

**Culture of primary rat liver cells**

Primaty liver cells were obtained by using a modified method as previously reported.[43] Primary liver cells were cultured for 4 or 21 days in 6-well culture dishes.

**Culture of primary mouse peritoneal macrophages**

Peritoneal macrophages were collected from TLR4KO mice and WT mice as described previously.[44] Peritoneal macrophages were cultured for 4 days in 6-well plates.

**Stimulation of cultured cells**

Primary liver cells cultivated for 4 days, which contain hepatocytes, were stimulated with IL-6 for 12 hours, or were used unstimulated as control cells. Culture medium containing Hb was prepared by adding Hb into the culture medium. Primary liver cells cultivated for 21 days, which contain macrophages and fibroblasts, were stimulated with Hb by changing the medium with Hb-containing medium prepared as described above and incubating for 12 hours. Peritoneal macrophages cultivated for 3-4 days were stimulated with Hb and unstimulated macrophage cultures were used as control.

**RNA extraction and real time RT-PCR**

Total RNA was isolated from the materials described above using Trizol and cDNA was synthesized. To create the plasmids used for the standard, rat hepcidin, BMP-6, IL-1β and IL-6, mouse IL-1β, and IL-6 mRNA were amplified using the primer pairs shown in Table S1 and the primers as reported previously.[34,45,46] The absolute copy number of each mRNA was also measured by a LightCycler instrument.

### Statistical analysis

Statistical assessment was performed by a nonpaired Student t test or one way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. Correlations between log-transformed hepcidin mRNA copy number and blood Hb concentration were evaluated by linear regression analysis. Data were analyzed using Pearson's correlation coefficient and Fisher's Z-transformation test

## Results

### Organ weight and histological finding

On day 4 after ligation, the mean body weight of rats with LC decreased compared with sham-operated rats but started to recover from day 7 (Fig. 1A). On days 28, 42, and 84, rats with LC were active and with good appetites. The mean liver weight/body weight ratio of rats with LC was significantly higher than that of sham rats at all time points (Fig. 1B). The mean spleen weight/body weight ratio of rats with LC was slightly but significantly higher than that of sham-operated rats (Fig. 1C) but the mean kidney weight/body weight ratio and histology of kidney tissues were not notably different between the 2 groups (data not shown). The mean spleen weight/body weight ratio of rats with HA injected with PHZ was markedly significantly higher than that of sham-operated rats (Fig. 1C).

Macroscopic- and microscopic examination of livers of rats with LC showed severe LC from day 4 to day 84 (Fig.s S1A and S1B). Many hemosiderin-laden macrophages were found in hematoxylin/eosin-stained sections

of congestive rat livers (Fig. S1B). The number of $CD68^+$ macrophages and MHC Class $II^+$ cells that were associated with inflammation in congestive livers were markedly higher compared with those in liver of sham-operated rats (Fig.s S1C and S1D). Liver and spleen of sham-operated rats and rats with BA had no abnormal histological findings (data not shown).

**Anemia and serum iron**

Rats with LC had more severe anemia than sham-operated rats at all time points (Fig. 2A). Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) of rats with LC markedly decreased starting from day 28 and microcytic hypochromic anemia was observed (Fig.s 2B and 2C). Moreover, serum iron concentrations and transferrin saturation in LC rats were decreased (Fig. 2D, 2E). These findings show a state of iron deficiency. On the other hand, rats with BA and rats with HA had anemia immediately after the treatment but recovered from anemia 14 days after the treatment (Fig. 2A). There were no decreases in serum iron concentration and transferrin saturation in rats with BA and rats with HA at all the time points (Fig.s 2D and 2E).

**Expression of hepcidin, BMP-6, IL-6, and serum hepcidin level**

Hepcidin expression is known to be markedly decreased in anemia and iron deficiency.[28,47] Hepcidin expression in livers of sham-operated rats, rats with BA, and rats with HA was markedly decreased (Fig. 3A, solid line). However, although

rats with LC had anemia and RID, hepcidin expression in these rats (Fig. 3A, dashed line) was not suppressed as much as in sham-operated rats, rats with BA, and rats with HA. On day 4, the serum hepcidin levels in rats with LC were significantly higher than those in sham-operated rats (Fig. 3B). On day 84, when the transferring saturation was the lowest and the morphology of the red blood cells was most notably microcytic and hypochromic, the serum hepcidin levels in rats with LC were not significantly lower than those in sham-operated rats (Fig. 3B), and serum levels of IL-6, which are inducer of hepcidin, in rats with LC were significantly higher than those in sham-operated rats (Fig. 3C). On day 84, the expression levels of IL-6 and BMP-6, which are inducer of hepcidin, in livers of rats with LC were significantly higher than those of sham-operated rats (Fig. 3D, 3E).

**Distribution of iron**

Many hemosiderin-laden macrophages were observed in livers of rats with LC (Fig. S2A, left side panel). Hemosiderin-laden cells were also observed in the spleens (Fig. S2A, middle side panel) of rats with LC but not in bone marrow at all (Fig. S2A, right side panel). Many hemosiderin-laden cells were observed in the spleen (Fig. S2B, middle side panel) and bone marrow (Fig. S2B, right side panel) of sham-operated rats. Hemosiderin-laden cells, which were faintly stained by Berlin-blue, were observed in the liver (Fig. S2C, left side panel), spleen (Fig. S2C, middle side panel), and bone marrow (Fig. S2C, right side panel) of rats with HA.

Hemosiderin-laden cells were not observed in the liver (Fig. S2D, left side panel), spleen (Fig. S2D, middle side panel), and bone marrow (Fig. S2D, right side panel) of rats with BA.

**Amount of iron in the liver**

SEM-EPMA analyses indicated that iron content in livers of rats with LC (Fig. 4B, right side panel) was elevated compared with sham-operated rats (Fig. 4B, left side panel). Iron content in hepatocytes surrounding marked LC lesions was also elevated. On the other hand, small quantities of iron were detected in the sinusoids in the liver of sham-operated rats.

**Induction of hepcidin and proinflammatory proteins in culture cells**

IL-6 significantly induced hepcidin expression in liver cells, consisting of mainly hepatocytes, after for 4 days in culture (Fig. 5A). Hb significantly induced IL-1β and IL-6 expression in liver cells, consisting of mainly macrophages and fibroblasts, after 21 days in culture (Fig. 5B). Induction of IL1β and IL-6 expressions in peritoneal macrophages cultivated from TLR4KO mice were decreased compared with those from WT mice. Hb appeared to induce IL-1β and IL-6 expressions via TLR4 (Fig. 5C).

## Discussion

**Hepcidin and anemia/RID in rats with LC**

Although bleeding during surgery is thought to be one cause of anemia in sham-operated rats and rats with LC, our data suggest that inappropriate expression of hepcidin by LC contributes to anemia and relative iron deficiency. The reasons are as follows. Anemia in sham-operated rats recovered immediately after surgery but anemia in rats with LC continued and gradually became microcytic and hypochromic. In addition, serum iron levels and transferrin saturation in rats with LC were continuously low, but those in BA and HA rats were rather high. These finding showed that rats with LC were continuously relative iron deficient. In addition, these data showed that BA and HA were associated with a dramatic decrease in liver hepcidin gene expression but anemia in rats with LC was not associated with decrease in expression and serum level of hepcidin. These results suggested that inappropriate expression of hepcidin due to LC contributed to RID and continuous RID caused chronic anemia. Hepcidin elevation leads to impaired utilization of iron from macrophage stores, reduced absorption of dietary iron, and a resulting hypoferremic state that impairs the delivery of iron to maturing erythroid cells in the bone marrow.[48] In the liver, spleen, and bone marrow of rats with HA in which hepcidin expression was dramatically decreased, hemosiderin-laden cells were faintly stained by Berlin-blue. On the other hand, hemosiderin-laden cells in liver and spleen of rats with LC, in which hepcidin expression was not decreased, were clearly stained by Berlin-blue but those in the bone marrow were not stained at all. Results of iron staining of tissues were also in accord with the explanation that inappropriate expression of hepcidin in rats with

LC impaired the utilization of iron from macrophage stores in liver and spleen, and the delivery of iron to the bone marrow.

**Mechamism of inappropriate expression of hepcidin in rats with LC**

We postulated that the reasons for inappropriate expression of hepcidin due to LC were increasing expression of BMP-6 and IL-6, which are hepcidin inducers,[30] in the liver (Fig. S3). BMP-6 is thought to be a protein controlled by intracellular iron contents.[30] Our results indicated that BMP-6 expressions in the livers of rats with LC and iron content in hepatocytes surrounding marked LC lesions were significantly higher than those of sham-operated rats. We surmised that iron released from destroyed erythrocytes moves into hepatocytes and the increase of intracellular iron content in hepatocytes induced BMP-6 expression, which then contributes to hepcidin expression. IL-6 is also thought to be important for induction of hepcidin. This study showed that IL-6 expression in the liver of rats with LC and serum IL-6 levels increased compared with that of sham-operated rats and IL-6-induced hepcidin expression in primary cultured hepatocytes. We speculated that heme protein was an important factor for induction of IL-6. Hb and free heme were reported to be a danger signal to activate the immune system.[40,41] Our previous study also demonstrated that free heme was a danger signal inducing expression of proinflammatory proteins.[38] The present study showed inflammation in several regions of the livers of rats with LC and Hb-induced expressions of IL-1β and IL-6 in macrophages and fibroblasts cultivated from liver.

Further, we postulated that TLR4 was an important receptor for induction of IL-1 and IL-6. A few studies reported that free heme and Hb induced proinflammatory proteins via TLR4[40,49] and our present study also showed decreased induction of IL-1 and IL-6 by Hb in peritoneal macrophages cultivated from TLR4KO mice. Many TLR4-positive cells were found in the sinusoids of the liver (data not shown). Previous reports have shown that Kupffer cells and hepatic stellate cells (HSCs) in the sinusoids of the liver play critical roles in liver injury via TLR4.[50-52] We hypothesize that Kupffer cells and HSCs expressing TLR4 are activated by Hb and free heme and produce IL-1 and IL-6 and then IL-6 contributes to the expression of hepcidin in hepatocytes (Fig. S3).

**Anemia in heart failure patients with LC**

The present study indicated that inappropriate hepcidin expression is induced by severe LC in a rat model. Clinical severe LC with heart failure is often caused by right heart failure associated with severe TR and pulmonary hypertension.[53,54] Anemia in such patients is suspected to be caused by bleeding from the gastrointestinal tract, albeit without the presence of a tumor or ulcer, because these patients are often being treated with anticoagulants and antiplatelet agents and venous pressure is high.[55] Alternatively, in right heart failure patients with artificial valve prostheses, mechanical hemolysis sometimes cause anemia.[56] Therefore, many clinicians have often suspected that anemia in severe right heart failure with LC is caused by bleeding or hemolysis. However, we suggested that

LC contribute to anemia and patterns of hepcidin expression are different between BA/HA and anemia by LC. We previously reported a case of a woman with severe TR with anemia and with a very high serum hepcidin level who recovered from anemia after valve replacement, which was accompanied by a decrease in the hepcidin level.[36] We propose that LC associated with heart failure contributes to RID and anemia, and does so via inappropriate expression of hepcidin.

**Serum hepcidin concentration in heart failure patient with anemia**

The role of hepcidin in anemia/RID with heart failure has been previously investigated by measuring serum hepcidin concentration.[32,57] Some studies negated the role of hepcidin as a cause of anemia in heart failure,[31,57] while other papers partially supported it.[32,58] Thus, interpretation of the role of serum hepcidin concentration in anemia/RID patients with heart failure remains unclear. We speculate that the difficulty of interpretation is due to many anemia-causing factors in heart failure patients and the hepcidin concentration influenced by the severity of anemia itself. Choi et al. recently reported that there was a significant parametric correlation between levels of blood Hb and levels of serum hepcidin in children with iron deficiency.[59] There is an interesting paper by Artunc et al. about the role of serum erythropoietin (EPO), another anemia-related hormone. They reported that there was a strong parametric correlation between severity of anemia and increase in EPO in patients without chronic kidney disease (CKD); however, it was gradually attenuated with increasing stages of CKD and was completely lost in

CKD stages 4 and 5.[60] They demonstrated that concentrations of serum EPO in percentiles corrected for the severity of anemia improved the diagnostic value of measuring EPO concentrations for diagnosing relative EPO deficiency and renal anemia. This present study demonstrated that there was a strong parametric correlation between severity of anemia and decrease in hepcidin in rats with BA and HA; however, a good correlation was not found in rats with LC. We propose that serum hepcidin concentration should be also evaluated after correction for the severity of anemia. In simple terms, a slight increase or failure to decrease in serum hepcidin concentration in a patient with distinct anemia should be judged as inappropriate expression of hepcidin. Thus, we propose that anemia/RID with inappropriate hepcidin expression and BA/HA in heart failure patients may be distinguished by serum hepcidin concentration corrected for the severity of anemia.

**Study limitation**

A limitation of this study is that this LC model by ligating the IVC is not a heart failure model. Therefore, it could not be ascertained if LC by heart failure can also contribute to anemia/RID. Further studies are needed to evaluate anemia/RID in a model of heart failure with LC, and in heart failure patients with LC.

**Conflict of interest:** none declared.

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**Figure legends**

Fig. 1

Organ weight of anemia model rats

(A) Body weight in rats with LC (closed circles) and sham-operated rats (open rhombuses). (B) Liver weight/body weight in rats with LC (closed circles) and sham-operated rats (open rhombuses). (C) Spleen weight/body weight in anemia model rats. Statistical assessment was performed by a nonpaired Student's t test or one-way ANOVA and Bonferroni's multiple comparison test. ***P < 0.001 vs sham-operated rats; *P < 0.05 vs sham-operated rats. †††P < 0.001 vs Sham Day 84; ††P < 0.01 vs Sham Day 84; †P < 0.05 vs Sham Day 84.

Fig. 2

Hematological and iron parameters in sham-operated rats (open rhombuses), rats with LC (closed circles), rats with HA (open circles), and rats with BA (open squares). (A) Hb in anemia model rats. (B) MCV. (C) MCH. (D) Serum iron concentration. (E) Transferrin saturation. Statistical assessment was performed by a nonpaired Student's t test. ***P < 0.001 vs sham-operated rats; **P < 0.01 vs sham-operated rats; *P < 0.05 vs sham-operated rats.

Fig. 3

Expression of liver hepcidin, BMP-6, IL-6, and serum hepcidin level in anemia model rats. (A) Expression of hepcidin in liver of anemia model rats. Expression of hepcidin in the liver of sham-operated rats (open rhombuses), rats with BA (open squares), and rats with HA (open circles) was markedly decreased. On the other hand, hepcidin expression in rats with LC (closed circles) was not suppressed as much as in sham-operated rats, rats with BA, and rats with HA. Solid line indicates the correlation between log-transformed hepcidin mRNA copy number and blood Hb concentration in sham-operated rats, rats with BA, and rats with HA. Dashed line indicates that in rats with LC. (B) Serum hepcidin concentration in rats with LC (closed circles) and sham-operated rats (open rhombuses). (C) Serum IL-6 concentration in rats with LC and sham-operated rats on day 84. (D) IL-6 expression in livers of rats with LC and sham-operated rats on day 84. (E) BMP-6 expression in livers of rats with LC and sham-operated rats on day 84. Statistical analysis was performed by a nonpaired Student t test. ***$P < 0.001$ vs sham-operated rats; **$P < 0.001$ vs sham-operated rats; *$P < 0.05$ vs sham-operated rats.

Fig. 4

SEM-EPMA analyses in a liver of a sham-operated rat (left panels) and a rat with LC (right panels) on day 84. (A) Distribution of amino nitrogen in the pathologic image. (B) Distribution of iron.

Fig. 5

Gene expression induced by IL-6 and hemoglobin in cultured cells. (A) Hepcidin expression induced by IL-6 in cells cultivated from liver for 4 days, consisting mainly of hepatocytes (n=3). (B) Expression of IL-1β (left) and IL-6 (right) induced by hemoglobin in cells from liver cultured for 21 days, consisting mainly of macrophages and fibroblast (n=4). (C) Expression of IL1β (left) and IL-6 (right) induced by hemoglobin in cultured peritoneal macrophages from WT mice and TLR4KO mice. These analyses were performed 3 times to confirm the results. Statistical analysis was performed by a nonpaired Student's t test or one-way ANOVA and Bonferroni's multiple comparison test. ***P < 0.001 vs non-stimulated cells; **P < 0.01 vs non-stimulated cells; †††P < 0.001 vs WT mice.

**Figure 1**

Figure 1

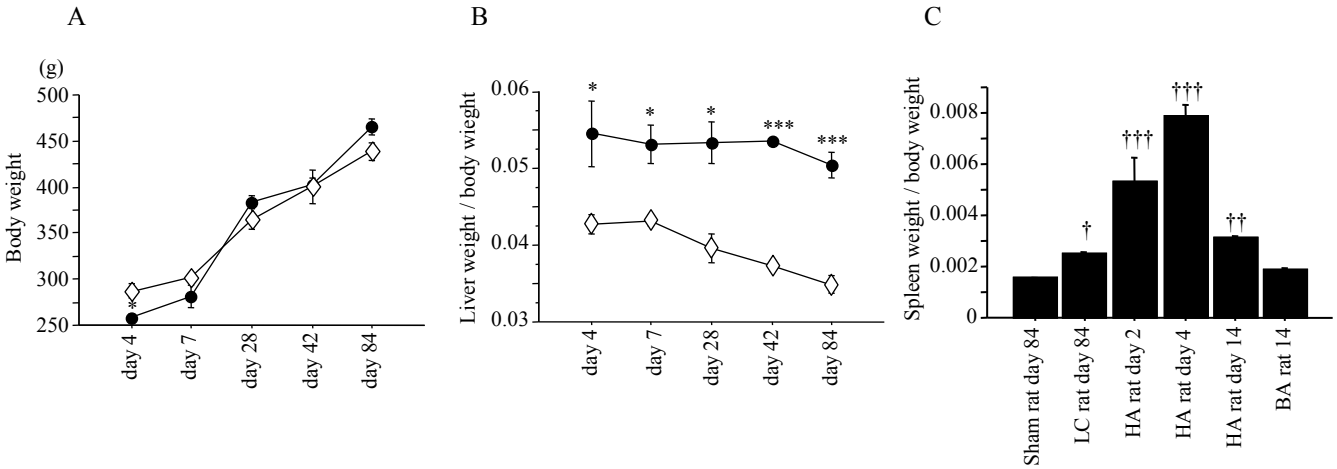


**Figure 2**

Figure 2

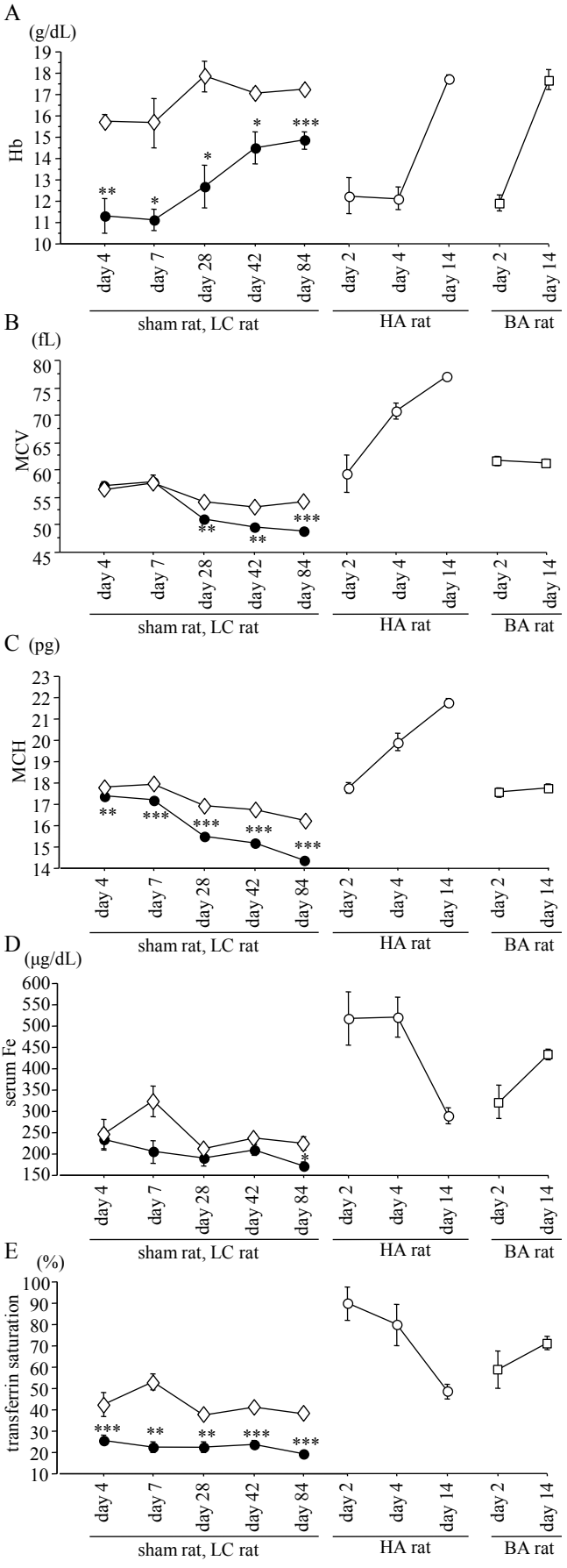


**Figure 3**

Figure 3

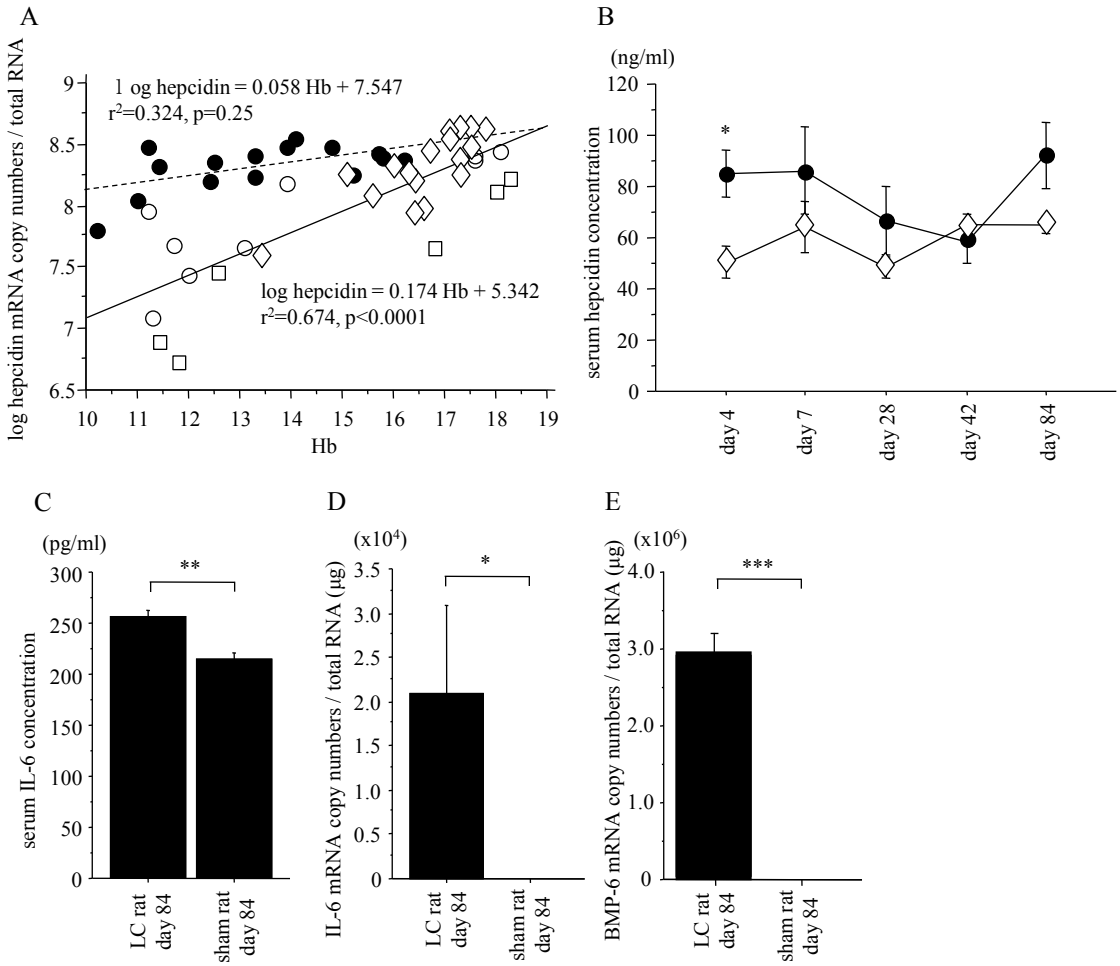


**Figure 4**

Figure 4

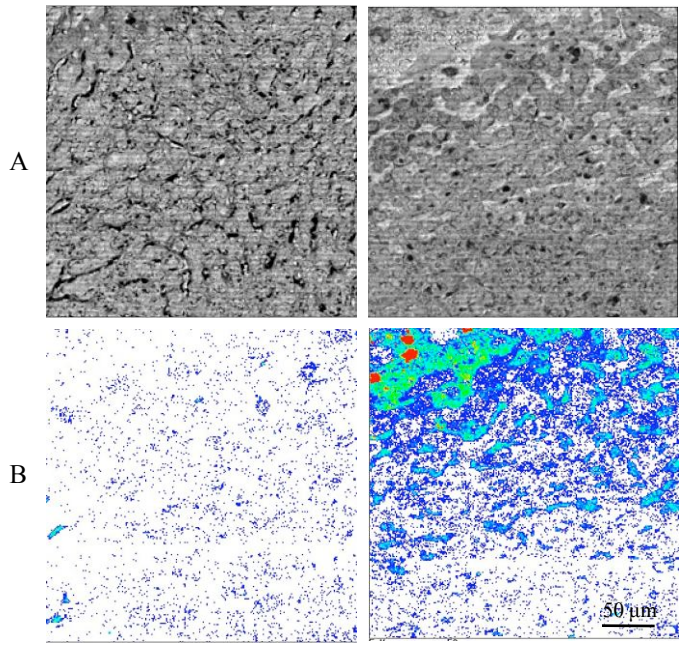
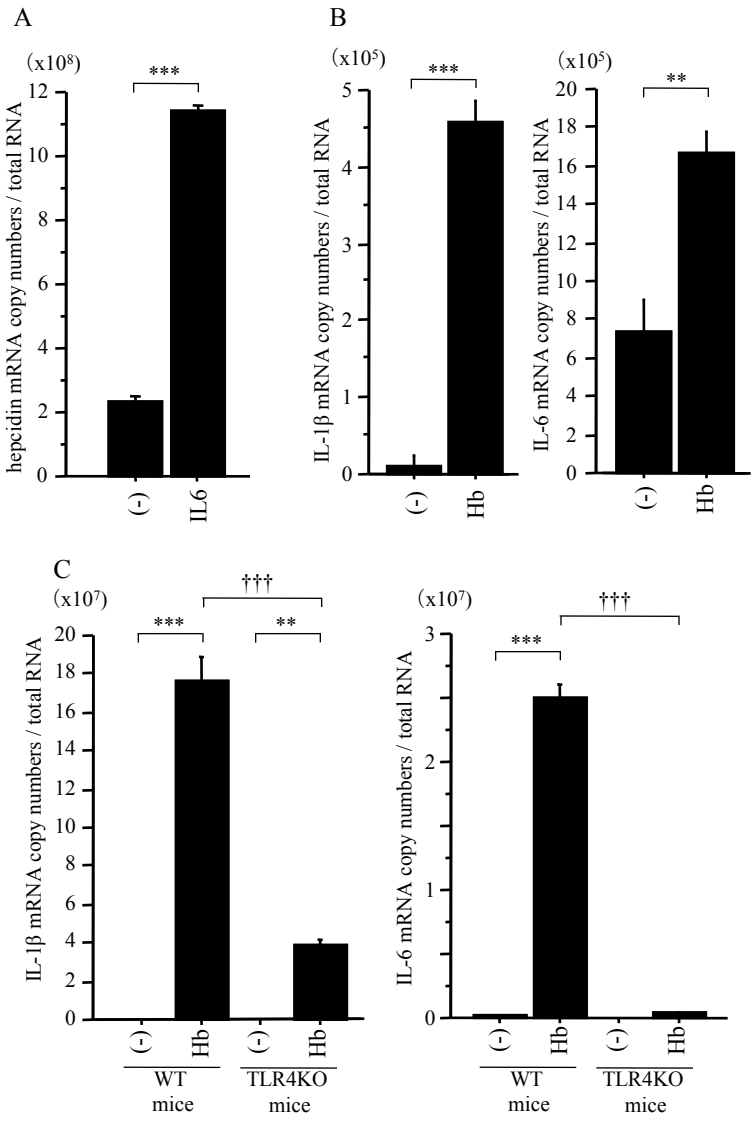


**Figure 5**

Figure 5



## Supplementary Methods

### Animal model

Male Lewis rats were obtained from Charles River, Japan (Atsugi, Kanagawa, Japan), and were maintained in our animal facilities until they reached 8 weeks of age (weighing 250 g) for use in creating anemia models. TLR4 knock-out (KO) mice were obtained from Oriental Bioservice Inc (Kyoto, Japan). TLR4KO mice and wild-type (WT) littermate mice, aged 8–10 weeks, and with a C57BL/6 background from Charles River, were used in the study. All animal experiments in our institute followed the guidelines for the care and use of laboratory animals published by the US National Institutes of Health.

### Rat models of liver congestion

All rats (n=38) were anesthetized with an intraperitoneal injection of medetomidine (150 μg/kg), butorphanol (2.5 mg/kg), and midazolam (2.0 mg/kg). LC was induced in rats (n=19) by ligating the abdominal portion of the IVC in the space between the diaphragm and the liver as previously reported.[41] After exposure of the liver through a mid-line incision, the diaphragm was pulled up and the falciform ligament was cut up to IVC. The IVC was tightly ligated with a silk thread, enclosing a glass tube (1.3 mm in diameter) in the ligature and the glass tube was immediately removed, to prevent complete occlusion. Sham-operated control rats (n=19) underwent the same operation without ligation. The animals were killed on day 4, day 7, day 28, day 42, and day 84

after the operation (n=3-5, each group). Liver, spleen and bone marrow from femurs were collected for gene expression and histological analysis.

**Rat models of bleeding anemia and hemolytic anemia**

Six rats were bled by PHL of 2 or 3 ml blood every 12 hours (3 times) as BA rats. The BA rats were killed on day 2 (n=3) and day 14 (n=3) after first PHL. Nine rats received intraperitoneal injection of PHZ (40 mg/kg) twice for 2 days as HA rats. The HA rats were killed day 2 (n=3), day 4 (n=3), and day 14 (n=3) after the first injection. Liver, spleen, and bone marrow from femurs were collected for gene expression analysis and histological analysis.

**Blood count and serum iron measurement**

*Blood count*

Peripheral blood samples were obtained from the right atrium. Red blood cell count, blood Hb concentration, and hematocrit, were determined using the automated XE-2100 analyzer (Sysmex, Kobe, Japan).

*Serum iron measurement*

Rat serum iron and unsaturated iron-binding capacity (UIBC) were determined using an automated system H7700 (Hitachi High-Tech, Tokyo, Japan). The total iron-binding capacity (TIBC) was calculated as the sum of serum iron and UIBC, and the percentage of transferrin saturation was calculated as serum iron/TIBC × 100.

**Serum hepcidin measurement**

Serum hepcidin concentrations were determined by an Enzyme-linked Immunosorbent Assay Kit for rat hepcidin (Uscn Life Science Inc., Wuhan, China).

**Serum IL-6 measurement**

Serum IL-6 concentrations were determined by an Enzyme-linked Immunosorbent Assay Kit for rat IL-6 (R&D Systems Inc, Minneapolis, MN, USA).

**Histology and immunohistochemistry**

Tissue samples were fixed at room temperature in 10% formalin. Samples from liver, spleen, and bone marrow were sequentially dehydrated through an alcohol series and embedded in paraffin. Four-μm thick sections were cut, deparaffinized in xylene, and dehydrated in descending dilutions of ethanol. After washing in 0.01 M phosphate-buffered saline (PBS), endogenous peroxidase activity was blocked by treatment for 30 min with peroxidase blocking solution (DakoCytomation Carpinteria, CA, USA). Specimens were treated with formic acid and incubated in phosphate buffer (pH 6.0) at 110°C for 10 min in an autoclave for antigen retrieval. Specimens were reacted with mouse anti-CD68 antibody (ED1) (Serotec Ltd, Oxford, UK) and mouse anti-MHC Class II antibody (OX6) overnight at room temperature. The slides were washed in DAKO wash buffer (DakoCytomation) 3 times. Immunodetection was performed using biotinylated anti-rabbit and anti-mouse immunoglobulins (LSAB2 kit

for use on rat specimens; DakoCytomation) followed by horseradish peroxidase-conjugated streptavidin and 3,3-diaminobenzidine (DAB) substrate (DakoCytomation). Each section from liver, spleen, and bone marrow was also stained with haematoxylin–eosin stain and Berlin blue stain for iron (Berlin blue staining set; WAKO Pure Chemical, Osaka, Japan).

**Electron probe microanalyzer**

The chemical composition and ultrastructure of the prepared samples were studied using scanning electron microscopy and electron probe microanalysis (SEM-EPMA) (EPMA1610, Shimadzu, Kyoto, Japan). Sections of appropriate thicknesses were prepared by use of glass-like carbon as a support. For element mapping, lithium fluoride was used and the x-rays were digitally analyzed (pixel size 1 μm). The pixel count of each axis was always 256 steps. Scan time per pixel was 0.45 second. Thus, the total time of one area scan was 0.45 second multiplied by the total number of pixel 256 x 256, which is equal to 29,491 seconds, or 8.2 hours. The accelerating voltage and beam current were 20 kV and 0.8 μA, respectively. The distribution of amino nitrogen in the pathologic image was also mapped for each sample. All of the sampling procedures by EPMA with WDS were computer-operated.

**Culture of primary rat liver cells**

Lewis rats that were anesthetized as described above were heparinized with 500 U heparin by intraperitoneal injection and the portal vein was cannulated and perfused

with prewarmed (37 °C) washing solution, calcium and magnesium-free Hank's Balanced Salt Solution (HBSS) containing 0.5 mM EGTA, 10 mM HEPES and 4.2 mM NaHCO3 (pH 7.2), at a rate of 10 ml/min for 10 min, and then with prewarmed (37 °C) collagenase solution, HBSS containing 10 mM HEPES and 4.2 mM NaHCO3 supplemented with 0.05% Type II collagenase (Invitrogen, Grand Island, NY, USA) and 50 μg/ml trypsin inhibitor (pH 7.5), at a rate of 10 ml/min for 10-20 minutes. The liver was removed into a sterile beaker containing 25 ml of collagenase solution and minced into small pieces by scissors.[42] Separated liver cells were obtained by forcing the small pieces of liver through a 200-gauge stainless steel mesh and 25 ml of cold Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was added into the resultant cell suspension. The suspension was filtered through a 125-μm stainless steel sieve and the filtrate was transferred into 50 ml conical tubes and centrifuged at 350 x g for 5 minutes at 4°C and then washed with DMEM 3 times with resuspension and centrifugation of the cell pellet each time. Primary liver cells thus obtained were cultured for 4 or 21 days in 35-mm 6-well culture dishes in Williams' E medium containing 10% FBS, 1 mM human insulin (Invitrogen, Tokyo, Japan), 1 mM dexamethasone, 10 mM nicotinamide, and 20 ng/ mL human EGF (R&D Systems Inc, Minneapolis, MN, USA), at 37°C in an atmosphere of 5% CO2/95% air.

**Culture of primary mouse peritoneal macrophages**

Thioglycollate-elicited peritoneal macrophages were collected from TLR4KO mice and WT mice by peritoneal lavage, 3-4 day after intraperitoneal injection of 3%

thioglycollate as described previously.[43] Peritoneal macrophages were cultured in DMEM containing 10% FBS in 6-well plates at 37°C in an atmosphere of 5% CO2/95% air for 4 days.

**Stimulation of cultured cells**

Primary liver cells cultivated for 4 days (n=3), which contain hepatocytes, were stimulated with 50 ng/mL human IL-6 (R&D Systems Inc) for 12 hours, or were used unstimulated as control cells. Culture medium containing 50 μM Hb (WAKO Pure Chemical) was prepared by adding Hb into the culture medium and passage through a sterile 0.22 μm filter. Primary liver cells cultivated for 21 days (n=4 wells), which contain macrophages and fibroblasts, were stimulated with Hb by changing the medium with Hb-containing medium prepared as described above and incubating for 12 hours. Peritoneal macrophages cultivated for 3-4 days (n=4 wells) were stimulated with 50 μM Hb and unstimulated macrophage cultures were used as control.

**RNA extraction and real time RT-PCR**

Total RNA was isolated from the materials described above using Trizol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 to 5 μg of total RNA with random primers and murine Moloney leukemia virus reverse transcriptase. To create the plasmids used for the standard, rat hepcidin, rat BMP-6, rat IL-1 $\beta$, rat IL-6, mouse IL-1 $\beta$, and mouse IL-6 mRNA were amplified using the primer pairs shown in Table S1 and the primers as reported previously.[33, 44, 45] PCR amplified cDNAs were inserted

directly into the pGEM recombinant plasmids and were isolated following transformation into Escherichia coli JM109-competent cells using a MagExtractor plasmid kit (Toyobo, Osaka, Japan). The absolute copy number of each mRNA was also measured by quantitative real-time RT-PCR using a LightCycler instrument (Roche Diagnostics, Tokyo, Japan) using the same primers and SYBR Premix Ex Taq (Takara, Otsu, Japan). After an initial denaturation step of 10 minutes at 95°C, a 3-step cycling procedure (denaturation at 95°C for 10 s, annealing at 62°C for 10 s and extension at 72°C for 13 s) was used for 45 cycles. The absolute copy numbers of particular transcripts were calculated by LightCycler software using a standard curve approach. Gene expressions of 5 samples in a rat liver preparation were averaged.

**Statistical analysis**

Statistical assessment was performed by a nonpaired Student t test or one way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. The differences were considered significant at $p < 0.05$. The data obtained from organ weight, blood examination, quantitative RT-PCR, and ELISA were expressed as mean ± SEM. Correlations between log-transformed hepcidin mRNA copy number and blood Hb concentration were evaluated by linear regression analysis. Data were analyzed using Pearson's correlation coefficient and Fisher's Z-transformation test

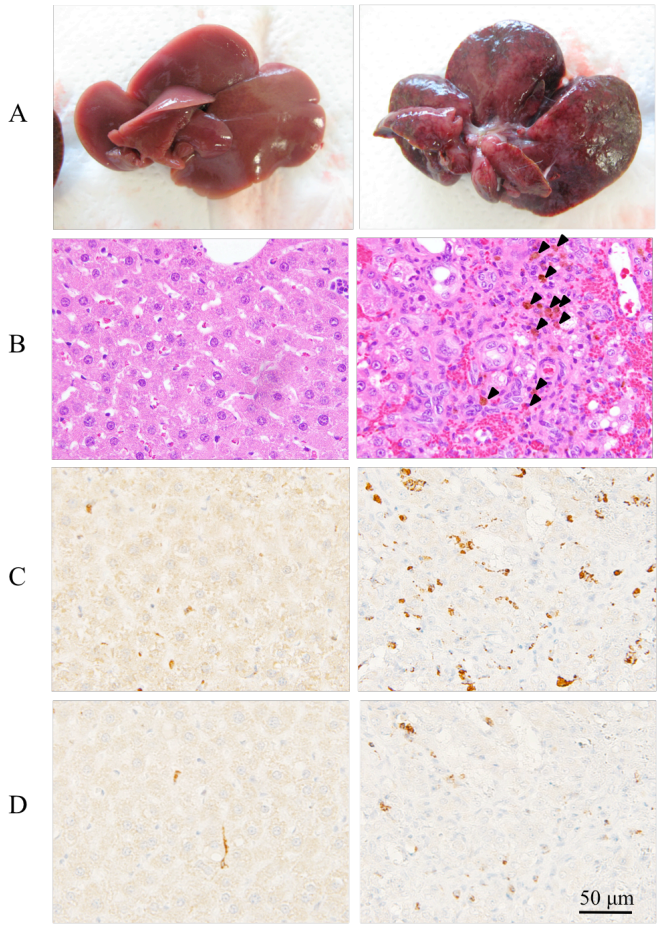



Fig. S1

Macroscopic and microscopic examination of liver in a sham-operated rat (left panels) and a rat with LC (right panels) on day 84. (A) Macroscopic findings of the liver. (B) Microscopic findings of the liver sections stained with hematoxylin-eosin. Arrow heads indicate hemosiderin-laden macrophages. (C) CD68 staining. (D) MHC Class II staining.

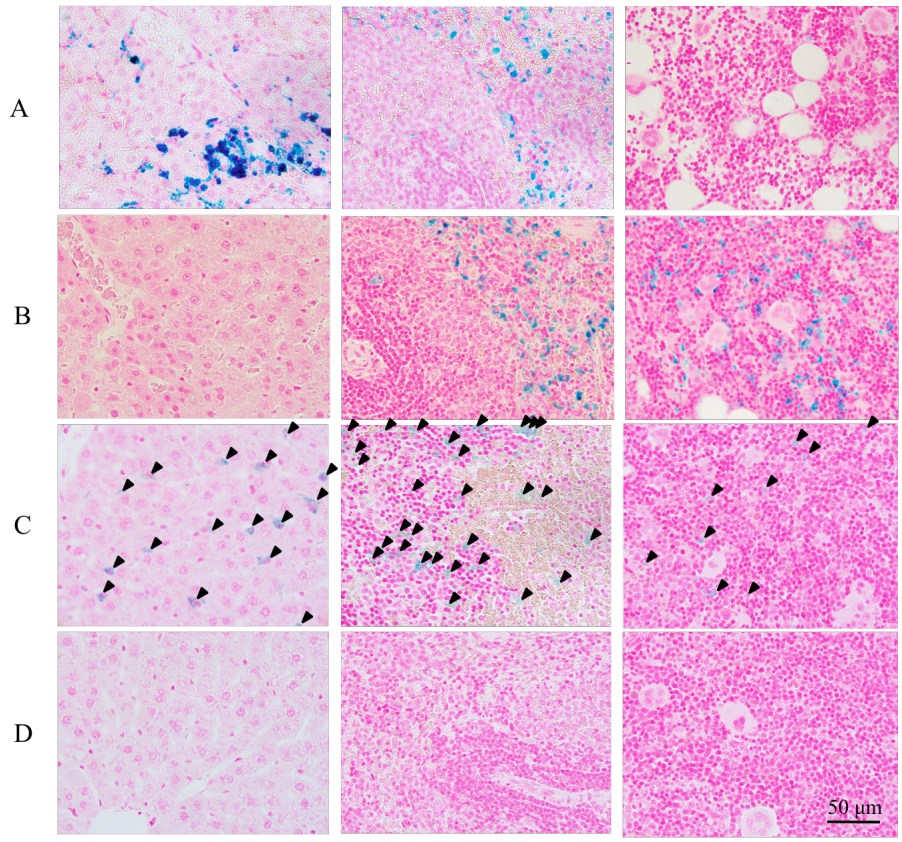



Fig. S2

Microscopic finding of tissue sections stained with Berlin blue for iron. (A) Sections from a rat with LC on day 84. (B) Sections from a sham-operated rat on day 84. (C) Sections from a rat with HA on day 4. (D) Sections from a rat with BA on day 2. Arrow heads indicate hemosiderin-laden cells, which were faintly stained by Berlin-blue, in sections from a rat with HA. Left panels are from livers. Middle panels are from spleens. Right panels are from bone marrow.

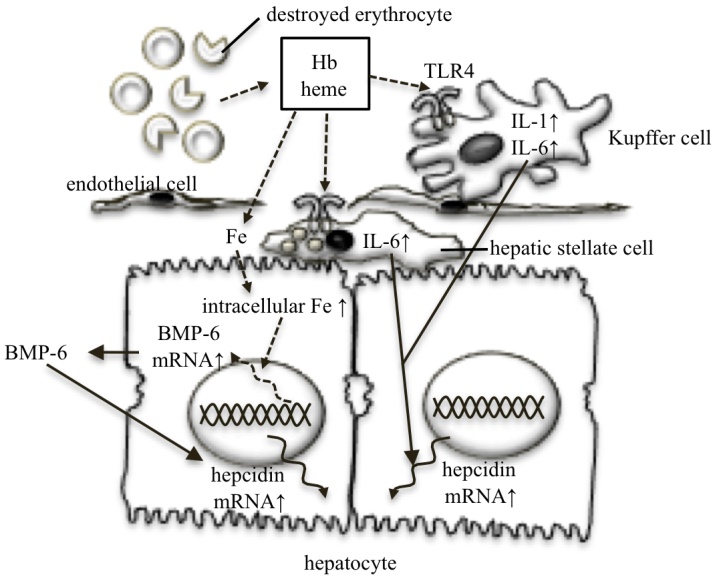



Fig. S3

Putative mechanism of upregulation of hepcidin in liver of rats with LC. Hb and free heme released through destruction of erythrocytes activate Kupffer cells and hepatic stellate cells via TLR4 and induce IL-1 and IL-6 expression. IL-6 induces hepcidin expression in hepatocytes. Iron released through destruction of erythrocytes moves into hepatocytes and the increase of intracellular iron content in hepatocytes induces BMP-6 expression, which then results in expression of hepcidin

Table 1. List of Primers for quantitative RT-PCR

| | Sense Primer | Antisense Primer |
|---|---|---|
| rat BMP-6 | 5'-acatcacagcaactagcaatctgt-3' | 5'-tcttgcaagctgtttttaactcac-3' |
| mouse IL-1 $\beta$ | 5'-caacaagtgatattctccatgagc-3' | 5'-atggtgaagtcaattatgtcctga-3' |
| mouse IL-6 | 5'-ggcaattctgattgtatgaacaac-3' | 5'-gaaattggggtaggaaggactatt-3' |