Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Repression of Repulsive Guidance Molecule C during Inflammation Is Independent of Hfe and Involves Tumor Necrosis Factor- α

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Genetic iron overload, or hemochromatosis, can be caused by mutations in *HFE*, hemojuvelin, and hepcidin genes. Hepcidin, a negative regulator of intestinal iron absorption, is found to be inappropriately low in both patients and in animal models, indicating that proper control of basal hepcidin levels requires both hemojuvelin and HFE. In mice, repulsive guidance molecule c (*Rgmc*, the hemojuvelin mouse ortholog) and hepcidin levels are transcriptionally regulated during inflammation. Here, we report that basal *Rgmc* levels in *Hfe*-deficient mice are normal and that these mice retain the ability to suppress *Rgmc* expression after lipopolysaccharide (LPS) challenge. Thus, *Rgmc* regulation by LPS is *Hfe*-independent. The response of *Rgmc* to LPS involves signaling through toll-like receptor 4 (*Tlr4*), because *Tlr4*-deficient mice do not show altered *Rgmc* expression after LPS administration. We further show that tumor necrosis factor- α , but not interleukin-6, is sufficient to cause *Rgmc* down-regulation by LPS. These results contrast with previous data demonstrating that hepcidin levels are directly regulated by interleukin-6 but not by tumor necrosis factor- α . The regulation of iron-related genes by different cytokines may allow for time-dependent control of iron metabolism changes during inflammation and may be relevant to chronic inflammation, infections, and cancer settings, leading to the development of anemia of chronic disease. (*Am J Pathol* 2007, 170:497–504; DOI: 10.2353/ajpath.2007.060437)

Iron is essential for life, but its excess is toxic because of its potential participation in the generation of reactive

oxygen species. Heavy iron overload is observed in primary (hereditary) and secondary hemochromatosis. Excessive intestinal absorption, as it occurs in hereditary hemochromatosis (HH), leads to iron deposition in the parenchymal cells of various organs, such as the liver, heart, and pancreas. In the liver, common complications include progressive fibrosis resulting in cirrhosis and hepatocellular carcinoma.¹ In secondary hemochromatosis, as seen in patients with thalassemia receiving regular transfusion therapy, excess iron initially accumulates in reticuloendothelial macrophages and, later, when reticuloendothelial iron stores saturate, iron deposition starts to increase in parenchymal tissues such as the endocrine glands, liver, and myocardium. Cardiac iron deposition produces arrhythmias, systolic and diastolic dysfunction, and congestive heart failure.²

Recently, several genes have been identified that, when mutated, can elicit HH. HH type 1, the most common form, is a late-onset autosomal recessive disease caused by mutations in *HFE*, which codes for a ubiquitously expressed^{3,4} major histocompatibility complex class I-like molecule.⁵ *HFE* requires β 2-microglobulin (β 2m) for appropriate cell surface expression,⁶ and in fact both *Hfe*^{-/-} and β 2m^{-/-} mice recapitulate human HH and develop iron overload in the liver because of iron hyperabsorption in the duodenum.^{7–9} *HFE* influences iron homeostasis through its binding to transferrin receptor 1 (TfR1), which is part of the major cellular iron uptake pathway, and by reducing the affinity of TfR1 for transferrin (Tf), thus competing with Tf binding.^{10,11}

Juvenile hemochromatosis (JH) or type 2 hemochromatosis, a rare autosomal disease, shares several fea-

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tures with HFE hemochromatosis. However, because the rate of iron accumulation is much faster than in the classical form, all of the clinical manifestations develop earlier, typically in the first or second decade of life. In JH, the most prominent clinical features are heart failure and endocrine manifestations.¹² JH has been shown to have two causative factors: loss of function mutations in hepcidin (HAMP)¹³ or hemojuvelin (*HJV*)¹⁴ genes.

Hepcidin is a hormone discovered independently by three groups as a mouse peptide expressed in response to iron levels and lipopolysaccharide (LPS)¹⁵ and as a human antimicrobial peptide of the β -defensin family found in urine¹⁶ and blood.¹⁷ In all these reports, hepcidin was found to be highly expressed in the liver. Furthermore, mice deficient in hepcidin are iron-overloaded¹⁸ whereas, conversely, transgenic mice overexpressing hepcidin are severely anemic,¹⁹ suggesting that hepcidin is a negative regulator of iron absorption. Hepcidin expression in the liver is regulated by iron levels and inflammation,²⁰ and its mechanism of action includes posttranslational regulation of the cellular iron exporter ferroportin 1 (Fp1) in intestinal epithelial cells and macrophages.²¹

Comparatively, much less is known about *HJV*. *HJV* encodes for a glycosylphosphatidylinositol-anchored protein that has a partial von Willebrand factor type D domain and an Arg-Gly-Asp motif, usual in the structure of integrins. *HJV* is uniformly expressed in skeletal muscle and is present selectively in periportal hepatocytes.²² *HJV* shares high similarity with repulsive guidance molecules (RGMs), which are involved in axonal guidance.²³ In the mouse, the Rgm family comprises three members, *Rgma*, *Rgmb*, and *Rgmc*, the mouse ortholog of *HJV*. In fact, *Rgmc*-deficient mice, like hepcidin-deficient mice, develop severe hemochromatosis, with deposition of excess iron, mostly in parenchymal cells of the liver, heart, and pancreas.^{22,24}

A common feature in both HFE-linked HH and JH caused by *HJV* mutations is lower than expected hepcidin basal levels.^{14,25} Although it is now clear that *Rgmc* participates in the maintenance of iron homeostasis, it is not yet fully understood how the gene is regulated, through which signaling pathways, and whether there is any relationship between *Rgmc* and HFE for the control of hepcidin basal levels.

In this study, to gain new insights into the regulation of *Rgmc*, we used mouse models of HH (*Hfe*^{-/-} and $\beta 2m$ ^{-/-}) and toll-like receptor 4 (Tlr4)-deficient mice to investigate adaptive changes of *Rgmc* levels. In addition, the role of individual cytokines, namely, interleukin (IL)-6 and tumor necrosis factor (TNF)- α , in the regulation of *Rgmc* was examined both *in vivo* and *in vitro*.

Materials and Methods

Animals

All procedures were performed in accordance with Canadian Council on Animal Care guidelines after approval by the institutional Animal Care Committee of the Centre Hospitalier de l'Université de Montréal. *Hfe*^{-/-} mice were

kindly provided by Dr. Nancy C. Andrews, Howard Hughes Medical Institute and Harvard Medical School, Children's Hospital, Boston, MA, in the 129/SvEvTac background⁹ and were backcrossed onto the C57BL/6 (B6) background for 10 generations (N10). C57BL/6 controls, $\beta 2m$ ^{-/-} (C57BL/6 background, N11), B6.129S6-*Il6*^{tm1Kopf} (IL-6-deficient; C57BL/6 background, N11), and the C3H substrains, C3H/HeOuj (wild type or WT) and C3H/HeJ (Tlr4-deficient), were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All animals were housed in a light- and temperature-controlled environment with free access to tap water and food.

Animal Treatments

Control mice were fed a commercial diet (Teklad Global 18% protein rodent diet; Harlan Teklad, Madison, WI). Dietary iron overload was produced by giving 8-week-old mice the same commercial diet supplemented with 2.5% (w/w) carbonyl iron (Sigma-Aldrich, St. Louis, MO) for 2 weeks. Iron deprivation was induced by feeding the mice the same commercial diet deficient in iron for 2 weeks.

Ten-week-old mice were injected with saline (LPS; *Escherichia coli* serotype 055:B5, 5 mg/kg^{26,27} or 25 mg/kg²⁸ i.p.; Sigma-Aldrich), recombinant mouse IL-6 (1 μ g i.p.^{29,30}; Cederlane Laboratories Ltd., Hornby, ON, Canada) or recombinant mouse TNF- α (1 μ g i.p.³⁰; Cederlane). Control mice were similarly injected with an equivalent volume of sterile saline solution. Livers were excised 6, 17, 24, and 48 hours after LPS administration and 3 and 6 hours after IL-6 or TNF- α injections.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Tissue samples were stored in RNALater (Qiagen, Mississauga, ON, Canada), and total RNA was extracted with Trizol reagent (Invitrogen, Burlington, ON, Canada). RT was performed with the Omniscript RT kit (Qiagen), using random hexamers and RNase inhibitor (Invitrogen). mRNA levels of *Rgmc*, β -actin, and *hepcidin* were measured by real-time PCR in a Rotor Gene 3000 real-time DNA detection system (Montreal Biotech Inc., Kirkland, QC, Canada) with the QuantiTect SYBR+Green I PCR kit (Qiagen).³¹ The following primers were used: β -actin 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGTGTGAA-GGTCTCAAA-3'; *Rgmc* 5'-AATTCACACATGCCGTGT-C-3' and TCAAAGGCTGCAGGAAGATT-3'; *hepcidin* 5'-AGAGCTGCAGCCTTTGCAC-3' and 5'-GAAGATGCAG-ATGGGGAAGT-3'. Expression levels were normalized to the housekeeping gene β -actin.

Hepatocyte Isolation and Treatments

Hepatocytes were isolated from adult mouse livers according to a procedure already described.³² Isolated cells were resuspended in Williams' E media with 5% fetal bovine serum and seeded on collagen-coated plates at a density of 2.4×10^5 cells per well. After 3 hours, unat-

tached cells were removed, and the medium replaced by fresh medium. Twenty-two hours after isolation, the medium was changed and replaced by fresh medium alone or medium containing IL-6 (20 ng/ml), TNF- α (20 ng/ml), or LPS (100 ng/ml) for 24 hours.

Serum Iron (SI) and Transferrin Saturation (TS) Measurements

SI, total iron binding capacity, and TS were assessed by colorimetric assay with the Kodak Ektachem DT60 system (Johnson & Johnson, Ortho Clinical Diagnostics, Mississauga, ON, Canada).³¹

Measurements of Tissue Iron Concentration

Liver iron concentrations were assessed by acid digestion of tissue samples, followed by iron quantification with atomic absorption spectroscopy.³³

Statistical Analysis

Student's *t*-test (unpaired, two-tailed) was applied for comparison between two groups. Comparisons between more than two groups were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison test.

Results

Effects of Iron Levels on *Rgmc* Expression

Feeding mice on diets with different iron content resulted in statistically significant differences in body iron levels, as evidenced by measuring liver iron concentrations (Figure 1A). Mice fed the low-iron diet had a 2.5-fold reduction in liver iron concentrations compared to mice fed the standard diet, whereas animals kept on the high-iron diet had an approximately twofold increase in hepatic iron stores. Hepatic *Rgmc* mRNA levels, however, did not differ among mice subjected to the different diets (Figure 1B). Thus, changes in body iron levels through diet do not affect *Rgmc* quantitatively at the mRNA level. In contrast, and as expected,^{15,34} hepcidin levels were clearly regulated by iron stores, increasing with iron loading and decreasing when the dietary iron level was low (Figure 1C).

Hfe-Deficient Mice Show Normal Basal *Rgmc* Expression

Next, we investigated whether alterations in basal *Rgmc* levels in Hfe-deficient mice, namely $\beta 2m$ and Hfe knockout mice, contribute to inappropriate hepcidin levels found in these mice.^{25,35} As expected,^{7,9} $\beta 2m^{-/-}$ and Hfe $^{-/-}$ mice had approximately threefold more iron in the liver than WT mice (Figure 1D). Basal *Rgmc* and hepcidin mRNA levels in the livers of Hfe-deficient mice were similar to those in WT mice (Figure 1, E and F). Because,

Figure 1. Liver iron concentration, hepatic *Rgmc*, and hepcidin expression in control, iron-deficient, iron-loaded, and HH mouse models. Liver iron concentration was measured by atomic absorption spectroscopy (A and D). *Rgmc* and hepcidin mRNA expression were quantified by real-time RT-PCR (B, C, E, and F). In A–C, mice were fed for 2 weeks with an iron-deficient diet (–Fe), standard diet (Std) or iron-enriched diet (+Fe). In D–F, WT, Hfe $^{-/-}$, and $\beta 2m^{-/-}$ mice were fed Std. The *Rgmc*/β-actin $\times 10^2$ and hepcidin/β-actin ratios are shown. The animals were 10 weeks old. The results are presented as means $\pm$ SEM, *n* = 6 mice per group. Statistical analysis was performed by one-way analysis of variance. n.s., not significant.

unlike hepcidin, *Rgmc* mRNA levels were not regulated by iron stores (Figure 1B), this indicates that *Rgmc* was expressed at appropriate basal levels in the liver of Hfe-deficient mice.

LPS Represses *Rgmc* Expression in Both the Liver and Heart

To investigate changes in iron parameters and *Rgmc* expression in response to inflammation, we initially determined the effect of a single LPS administration at various times up to 48 hours. SI and TS were significantly lower for up to 24 hours after LPS challenge, returning to normal or even to higher than normal levels by 48 hours (Figure 2, A and B), indicating that mice become hypoferremic, at least during the first 24 hours. Hepatic *Rgmc* mRNA levels decreased significantly by 6 hours to 9.7% that of the control value and, in contrast to hypoferrmia, the reduction persisted for 48 hours, albeit a modest recovery was observed after the lowest point at 6 hours (Figure 2C).

Because hepatic *Rgmc* expression was down-regulated in the mouse liver after LPS administration, we investigated whether *Rgmc* expression was altered in the heart, which also expresses *Rgmc* and is an organ severely affected in JH.¹² As in the liver, LPS treatment caused an ~50% decrease in *Rgmc* mRNA level in the heart that persisted for 48 hours (Figure 2D). Thus, the LPS-induced acute phase response reduces *Rgmc* expression in both the mouse liver and heart.

Rgmc Repression by LPS Is Independent of Hfe

Next, to assess whether changes in *Rgmc* mRNA expression are appropriately elicited in Hfe-deficient mice, we

Figure 2. SI, TS, and *Rgmc* mRNA levels in the liver and heart at various times after LPS administration. SI (A) and TS (B) were measured at 0, 6, 17, 24, and 48 hours after LPS administration ($n = 4$ mice per group per time). *Rgmc* mRNA expression was quantified by real-time RT-PCR in the liver (C) and heart (D). The animals were 10 weeks old. The *Rgmc*/β-actin $\times 10^2$ ratio is shown. The results are presented as means $\pm$ SEM. Statistical analysis was performed by one-way analysis of variance: n.s., not significant; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with 0 hours.

measured *Rgmc* mRNA expression in *Hfe*^{-/-} and *β2m*^{-/-} mice after LPS administration. As shown in Figure 3, *Hfe*-deficient mice responded, like WT mice, by dramatically down-regulating their hepatic *Rgmc* expression (Figure 3A) and up-regulating hepcidin (Figure 3B). This demonstrates that the ability to down-regulate *Rgmc* during the acute phase response remains intact in the absence of functional *Hfe*.

Suppression of *Rgmc* Expression by LPS Involves Signaling through *Tlr4*

Pathogen-associated molecular patterns (PAMP), including LPS, are recognized by TLRs, which, upon activation,

Figure 3. Hepatic *Rgmc* and hepcidin mRNA levels in HH mouse models after LPS administration. Hepatic *Rgmc* (A) and hepcidin (B) mRNA expression was quantified by real-time RT-PCR in WT, *Hfe*^{-/-}, and *β2m*^{-/-}, saline (control, Ctrl)-, and LPS-treated (LPS) mice, 6 hours after treatments. The animals were 10 weeks old. The *Rgmc*/β-actin $\times 10^2$ and hepcidin/β-actin ratios are shown. The results are presented as means $\pm$ SEM, $n = 5$ to 6 mice per group. Statistical analysis was performed by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the controls.

Figure 4. SI, TS, *Rgmc*, and hepcidin modulation by LPS in *Tlr4*-deficient mice. SI (A) and TS (B) were measured 6 hours after 5 mg/kg or 25 mg/kg of LPS administration. *Rgmc* (C) and hepcidin (D) mRNA expressions were quantified by real-time RT-PCR in the liver ($n = 4$ to 5 mice per group) and *Rgmc* in the heart (E; $n = 3$ mice per group) of WT and *Tlr4*-deficient (*Tlr4*^{-/-}) mice. The animals were 10 weeks old. The *Rgmc*/β-actin $\times 10^2$ and hepcidin/β-actin ratios are shown. The results are presented as means $\pm$ SEM. Statistical analysis was performed by two-way analysis of variance: n.s., not significant; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the saline-treated controls (Ctrl).

lead to the production of proinflammatory cytokines. LPS is a PAMP that is recognized by *Tlr4* and the CD14 co-receptor.³⁶ To establish whether hypoferrremia and *Rgmc* down-regulation observed during LPS-induced acute inflammation are dependent on the *Tlr4* pathway, we tested two C3H mouse substrains: mice deficient in *Tlr4* (C3H/HeJ) and WT (C3H/HeOuJ) mice. We observed higher SI and TS values in saline-treated mice from this mouse strain (C3H) compared to C57BL/6 mice (Figure 2, A and B). Strain to strain differences in SI and TS, with C57BL/6 mice having the lowest values, have been observed by others³⁷; thus, these differences are attributable most likely to strain variations.

Both WT and *Tlr4*-deficient mice developed hypoferrremia 6 hours after LPS treatment, as evidenced by decreases in SI, by 30% in WT and 32% in *Tlr4*-deficient mice at 5 mg/kg and by 72% in WT and 51% in *Tlr4*-deficient mice at 25 mg/kg doses of LPS (Figure 4A). TS decreased by ~40% in both mouse strains at 5 mg/kg and by 71% in WT and 60% in *Tlr4*-deficient mice at 25 mg/kg doses of LPS (Figure 4B). We found that *Rgmc* repression and hepcidin up-regulation in the liver were suppressed in *Tlr4*-deficient animals at both 5 mg/kg and 25 mg/kg doses of LPS (Figure 4, C and D). In addition, *Rgmc* down-regulation in the heart was repressed (Figure 4E). Thus, the pathways involved in *Rgmc* and hepcidin regulation during inflammation, but not the hypoferrremic response, is *Tlr4*-dependent.

TNF-α Is Involved in the Suppression of *Rgmc* Expression by LPS

The *Tlr4* signaling pathway leads to activation of nuclear factor (NF)-κB, which initiates the transcription of proin-

Figure 5. SI, TS, *Rgmc*, and hepcidin modulation by proinflammatory cytokines and LPS. SI (A) and TS (B) in saline (control, Ctrl)-, TNF- α -, IL-6-, and LPS-treated mice. Hepatic *Rgmc* (C) and hepcidin (D) mRNA expression was quantified by real-time RT-PCR. The animals were 10 weeks old. The *Rgmc*/ β -actin $\times 10^2$ and hepcidin/ β -actin ratios are shown. The results are presented as means $\pm$ SEM, $n = 4$ mice per group. E and F: Primary hepatocytes treated with TNF- α , IL-6, and LPS. The fold changes in *Rgmc* (E) and hepcidin (F) mRNA levels, normalized to β -actin, are shown. The results are presented as means $\pm$ SEM. Statistical analysis was performed by one-way analysis of variance: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the controls.

inflammatory cytokine genes such as *Tnf- α* and *Il-6*. Consequently, in *Tlr4*-deficient C3H/HeJ mice, LPS induces markedly less production of TNF- α and IL-6 in serum, as well as hepatic mRNA levels (data not shown), than in *Tlr4*-normal C3H/HeOuJ mice,^{27,36,38,39} suggesting that cytokines might be mediators of the LPS-induced fall in *Rgmc* mRNA levels. To determine whether these cytokines are able to decrease *Rgmc* mRNA and cause hypoferrremia *in vivo*, C57BL/6 mice were treated with 1 μ g of recombinant mouse IL-6 or TNF- α for 3 or 6 hours. As shown in Figure 5, A and B, TNF- α led to hypoferrremia, because SI and TS were reduced to similar levels, as observed after LPS administration. Likewise, as reported in humans⁴⁰ and rats,⁴¹ IL-6 caused hypoferrremia at earlier time points (90 minutes), with a 29% decrease in SI ($P < 0.0001$) and a 22% decrease in TS ($P < 0.001$). TNF- α and IL-6 were both able to suppress *Rgmc* mRNA, albeit less than LPS treatment. In fact, TNF- α induced a 65% decrease at 3 hours and 48% at 6 hours, whereas IL-6 caused only a 38% decline at 3 hours (Figure 5C). In contrast, hepcidin expression was up-regulated twofold by IL-6 at 3 hours but remained unaffected by TNF- α treatment (Figure 5D).

To verify whether IL-6 and TNF- α were able to target *Rgmc* expression directly on hepatocytes, primary cultured mouse hepatocytes were prepared from perfused livers and treated with the two different cytokines. As illustrated in Figure 5E, exogenous TNF- α decreased *Rgmc* mRNA to 38% that of control levels, whereas no significant response was observed in IL-6-treated hepatocytes. Differently, and as expected,⁴² hepcidin levels were up-regulated only by IL-6 (Figure 5F). These results show that TNF- α , but not IL-6, is sufficient for the repression of *Rgmc* mRNA levels in hepatocytes.

Figure 6. SI, TS, *Rgmc*, and hepcidin modulation in IL-6-deficient mice. SI (A) and TS (B) in saline (control, Ctrl)-, LPS-treated WT and IL-6-deficient (*Il-6*^{-/-}) mice. Hepatic *Rgmc* (C) and hepcidin (D) mRNA expression were quantified by real-time RT-PCR. The animals were 10 weeks old and were analyzed 6 hours after treatments. The *Rgmc*/ β -actin $\times 10^2$ and hepcidin/ β -actin ratios are shown. The results are presented as means $\pm$ SEM, $n = 7$ mice per group. Statistical analysis was performed by Student's *t*-test: n.s., not significant, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the controls.

IL-6 Is Not Required for *Rgmc* Down-Regulation in Response to LPS

To exclude further the possible requirement for IL-6 in *Rgmc* down-regulation during inflammation, we tested IL-6 knockout mice. As seen in Figure 6, A and B, IL-6-deficient mice developed hypoferrremia, as SI and TS, respectively, decreased to 42 and 45% of control levels. Furthermore, IL-6-deficient mice retained their capacity to mount a robust response to LPS by down-regulating *Rgmc* expression by 87% (Figure 6C). In contrast, and as reported previously,⁴³ IL-6-deficient mice presented an impaired hepcidin response, as observed in Figure 6D. Thus, although IL-6 is necessary for hepcidin regulation, it is not needed for the modulation of *Rgmc* expression during inflammation induced by LPS.

Discussion

Rgmc has recently emerged as a key molecule involved in iron metabolism along with hepcidin, whose expression in the liver is modulated by iron levels. Two recent studies, probing whether *Rgmc* is similarly regulated by iron levels, used parenteral iron loading as a model and found different outcomes depending on the treatment given. In fact, after a single injection of iron polysomal-tosate (600 mg/kg), no significant changes were observed in *Rgmc* expression.⁴⁴ However, a significant in-

crease in *Rgmc* expression was reported in a mouse model with daily iron-dextran injections for 5 days (1 mg of iron dextran/day).⁴⁵ Importantly, parenteral iron loading results in excess iron being accumulated in reticuloendothelial macrophages and Kupffer cells, whereas dietary iron loading, the model in our study, leads to iron loading predominantly in parenchymal cells of the liver,⁷ mimicking the pattern in HH and JH.⁴⁶ Our results indicate that *Rgmc* mRNA levels are not regulated by dietary iron deprivation or iron supplementation, ie, by varying amounts of iron concentration in hepatocytes. Thus, iron sensing by *Rgmc* does not seem to involve modulation of *Rgmc* mRNA expression levels.

Despite the fact that *Rgmc* mRNA levels are not regulated by iron, recent evidence emerging from *Rgmc* knockout mouse studies indicate that *Rgmc* plays an essential role in the iron-sensing pathway at the systemic level, through the control of basal hepcidin levels. In fact, *Rgmc* knockout mice have low basal hepcidin levels and develop severe iron overload in the liver, heart, and pancreas, as do JH patients.^{22,24} Eventually, the lack of *Rgmc* regulation by iron at the mRNA level does not exclude the possibility of posttranscriptional regulation, either at the cell membrane, intracellular compartments, or even as a secreted factor.⁴⁷

Besides iron levels, iron homeostasis is also altered during inflammation, which is characterized by the occurrence of hypoferrremia and the regulation of expression levels of iron metabolism-related genes, including *Rgmc*.⁴⁴ The response to LPS seems to be tissue-specific because *Rgmc* down-regulation is not seen in the skeletal muscle of mice treated with LPS but is evident in the liver.²² We further show that down-regulation of *Rgmc* expression also occurs in the heart, where hepcidin is as well expressed,³¹ and persists for up to 48 hours in both the liver and heart. This indicates that *Rgmc* is important not only in the control of iron metabolism at the systemic level, as it impacts intestinal iron absorption through the regulation of hepcidin levels,²² but also locally, in the heart. This could be pertinent because cardiomyopathy is considerably more frequent in JH than in HH.⁴⁶

We also explored the possible connection between Hfe and *Rgmc* because HH patients and Hfe-deficient mice,^{25,35,48,49} like *Rgmc* knockout mice,^{22,24} have lower than expected basal hepcidin levels. We investigated whether Hfe-deficient mice express appropriate basal *Rgmc* levels and are able to respond to LPS challenge. Our finding that both Hfe-deficient strains tested show basal *Rgmc* levels similar to WT mice, coupled with the observation that they retain the ability to down-regulate *Rgmc* after LPS administration, excludes any involvement of Hfe in *Rgmc* regulation and participation in the pathogenesis of classical HH. Thus, Hfe and *Rgmc* control of basal hepcidin expression occurs through two independent pathways. Further support for the existence of these pathways in the regulation of basal hepcidin mRNA levels comes from the findings that *Rgmc* knockout mice are unable to respond to an increase in iron stores by up-regulating their hepcidin expression²²; in contrast, *Hfe*^{-/-} mice seem to retain this capacity.^{35,50} Thus, it appears unlikely that Hfe lies downstream of the *Rgmc*-

Figure 7. Model showing the iron-sensing and inflammatory pathways for *Rgmc* and hepcidin regulation. Iron-sensing pathway: Hfe/ β 2m and *Rgmc* regulate basal hepcidin expression independently. Iron-sensing by *Rgmc* does not involve modulation of *Rgmc* mRNA expression levels, but *Rgmc* is necessary for the regulation of hepcidin levels through the iron sensing-pathway.²² Inflammatory pathway: mRNA *Rgmc* down-regulation by LPS involves signaling through Tlr4 and consequent production of TNF- α . Down-regulation of *Rgmc* suppresses hepcidin control through the iron-sensing pathway and allows hepcidin induction by IL-6.

dependent pathway for hepcidin regulation by iron, but Hfe may instead regulate basal hepcidin levels through another pathway, as summarized by the model in Figure 7.

The molecular signaling mechanism for *Rgmc* by LPS seems to involve Tlr4 because *Rgmc* down-modulation was abrogated in both the liver and heart of Tlr4-deficient mice treated with LPS. However, we have shown that Tlr4-deficient mice and mice lacking downstream elements, such as IL-6-deficient mice, are still able to respond with hypoferrremia, indicating that iron metabolism changes elicited by LPS may occur through both Tlr4- and non-Tlr4-dependent mechanisms.

Activation of the Tlr4-dependent signaling pathway ultimately results in the production of proinflammatory cytokines, including IL-6 and TNF- α , which are known to directly influence iron metabolism and provoke hypoferrremia. The mechanism by which IL-6 is believed to induce hypoferrremia is related to its ability to stimulate hepcidin expression,⁴⁰ a finding confirmed in this study (Figure 5D), which in turn causes iron sequestration in macrophages and reduces intestinal iron absorption⁵¹ by binding to Fp1 and inducing its internalization.²¹ However, there is ample evidence that TNF- α administration is also sufficient to cause hypoferrremia⁵² by increasing iron sequestration within macrophages.⁵³ A potential mechanism for TNF- α -induced iron sequestration in both the liver and spleen⁵⁴ can be linked to the ability of TNF- α to directly down-regulate Fp1 in a variety of cells, including macrophages,⁵⁵ hepatocytes,³⁵ and endothelial cells.⁵⁶ Thus, TNF- α can cause hypoferrremia by a mechanism independent of hepcidin induction.⁵⁴

We found that *in vivo* both TNF- α and IL-6 treatments led to down-regulation of *Rgmc* expression. However, when tested in primary hepatocyte cultures, only TNF- α was able to reduce *Rgmc* expression. Proinflammatory cytokines are known to induce each other's expression,⁵⁷ which may explain why, *in vivo*, IL-6 treatment had a

modest repressive effect on *Rgmc* expression. In addition, we further excluded IL-6 requirement for *Rgmc* regulation by demonstrating that IL-6-deficient mice retain the ability to repress *Rgmc* expression after LPS administration. Taken together, these results indicate that TNF- α , but not IL-6, is sufficient to induce *Rgmc* down-regulation in hepatocytes. The data contrast with those reported for hepcidin, in which IL-6, but not TNF- α , was shown to up-regulate hepcidin expression in primary hepatocytes,^{40,43} a finding that we reproduced in our study.

Regulation of the expression of genes involved in iron metabolism by different cytokines may allow for finer and possibly time-dependent control of iron metabolism changes during the response to inflammation. In fact, the signaling cascade triggered by LPS leads to time-dependent and concerted release of proinflammatory cytokines, which is initiated by transduction of the LPS signal across the cell membrane by Tlr4. This is followed by TNF- α production, which, as we demonstrate here, can directly elicit *Rgmc* down-regulation. The initial surge in TNF- α production is a prerequisite to the subsequent production of other mediators, including IL-1 β ⁵⁸ and IL-6.^{57,59} These two cytokines, in turn, directly modulate the expression of hepcidin in hepatocytes.^{40,43,60} Consistent with our previous findings that the inflammatory pathway overrides the iron-sensing pathway for hepcidin regulation,³⁵ *Rgmc* suppression during inflammation seems to be a prerequisite for the transcriptional regulation of hepcidin by cytokines (Figure 7). These results provide further support for the mechanistic view proposed by others that the iron-sensing pathway is switched off during inflammation, which has emerged from their observation that, in *Rgmc*-deficient mice, the cytokine-dependent pathway for hepcidin regulation remains functional while the iron-sensing pathway is inoperative.²²

In conclusion, we report that *in vivo* *Rgmc* mRNA levels are not regulated by hepatocyte iron stores and that their control during the inflammatory response is Hfe-independent. Furthermore, LPS-induced signaling involves the Tlr4 pathway and consequent production of TNF- α and IL-6, which are sufficient for *Rgmc* and *hepcidin* regulation, respectively. These findings provide important insights into the regulatory pathways impacting iron metabolism changes during inflammation that may also be relevant to other disease settings affecting iron metabolism.

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