

CNS Inflammation Induced by Lipopolysaccharide Up-Regulates Hepatic Hecpidin Expression by Activating IL-6/JAK2/STAT3 Pathway in Mice

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Abstract

Background: LPS triggers inflammation response in periphery, whether the infection in CNS induced by LPS ICV injection affected the peripheral iron metabolism was unknown, The current study was to find out whether LPS injected to the brain could regulate hepcidin expression in liver and peripheral iron metabolism.

Methods: Wide type mice (IL-6+/+) and IL-6-/- mice of 8-week-old were performed on ICV injection with LPS. After 6h, hepcidin expression in liver, as well as serum iron and transferrin saturation was detected and calculated, we also tested the IL-6/JAK2/STAT3 pathway in hepcidin regulation in liver of IL-6 knockout (IL-6-/- mice) and IL-6+/+ mice, AG490 as an inhibitor of JAK2 was used to confirm the effect of IL-6/JAK2/STAT3 pathway on hepcidin expression in liver.

Results: Hecpidin mRNA, IL-6 mRNA and protein expression in the liver of IL-6-/- mice was significantly lower than IL-6+/+ mice after LPS administration. IL-6 deficiency abolished the decrease of serum iron, transferrin saturation induced by LPS injection. IL-6 deficiency also abolished the decrease of Fpn1, increase of pSTAT3 and Ft-L protein in liver. AG490 significantly reduced the pSTAT3 protein and abolished the changes of Fpn1 and Ft-L expression induced by LPS in liver.

Conclusion: These finding provided further evidence that the effect of central inflammation on the hepatic hepcidin expression and peripheral iron metabolism.

Keywords: Hecpidin, LPS, IL-6-/- mice, STAT3, AG490

Introduction

Brain is immunological restrict area with the existence of BBB when peripheral tissue is suffered with infection [1]. The protection is destroyed with defective or dis-function of BBB. LPS triggered inflammatory reaction by injected into the lateral ventricle in the brain is markably induced by activation of astrocyte and microglia cells in brain and the release of inflammatory cytokines [2]. Several studies have clarified central nervous system infection induced peripheral inflammatory reaction and produced inflammatory cytokines in periphery [3], The mechanisms that involved in peripheral inflammatory reaction induced by CNS infection mainly include the HPA, the sympathetic nervous system, inflammatory cytokines and ROS in periphery [4-6]. LPS is not restricted in brain when injected into the lateral ventricle. Previous research has showed LPS injected by ICV to mice increased BBB permeability at 4h after the injection, then the changes in permeability of BBB also resulted in LPS leakage into the circulation in periphery [6].

Iron is necessary for many metabolic processes in CNS and periphery, It acts as the electron transfer and co-factor for many metabolic enzymes [7], however, abnormal high level of iron generates ROS and enhanced oxidative stress results in the cell death, therefore, maintaining normal iron content and iron homeostasis are important for metabolic balance and normal physiological function [8]. it has been well-demonstrated that hepatic hepcidin is the key mediator of iron homeostasis and iron metabolism disorders [9-11]. Hepcidin induces the degradation of Fpn1 protein and subsequently inhibits the release of iron into plasma from duodenal enterocytes [12, 13], therefore, Hepcidin inhibits the absorption of iron from dietary, moreover, it inhibits the recycling of iron from splenic, liver macrophages and senescent erythrocytes, as well as the hepatocytes [14, 15]. Hereditary hemochromatosis with iron abnormal overload is associated with hepcidin deficiency, however, elevated expression of hepcidin in inflammatory diseases contributes to the development of anemia with iron restricted. To date, the regulation of hepcidin is transcriptional. Iron status and inflammation in body are investigated to increase hepcidin expression in liver through BMP/SMAD signaling pathway. Nevertheless, JAK2/STAT3 pathway is known as the major pathway in the elevation of hepcidin transcription stimulated by inflammatory cytokines [16, 17]. LPS generates many inflammatory cytokines by recognizing specific TLR's receptor families, and subsequently stimulates an acute hypoferrremia mainly due to elevated expression of hepcidin in liver [18-20], Several cytokines including primarily IL-6 [21, 22], IL-1 [34], IL-22 [23] and Interferon α [24] have been

investigate to up-regulate hepcidin expression mediated by JAK2/STAT3 pathway [25–27].

In previous *in vitro* study, we have showed that LPS increased hepcidin expression in neuron mediated by activation of the IL-6/STAT3 signaling pathway in microglia [28] IL-6 is a multifunctional cytokine that regulates various aspects of the immune response, acute-phase reaction [29] and regulates expression of hepcidin respond to inflammation [30, 31]. Our previous *in vivo* study also has showed that IL-6 knockout significantly decreased the hepcidin expression and phosphorylation of STAT3 in the cortex and hippocampus, as well as Fpn1 and Ft-L with LPS ICV injection [44]. However, whether the infection in CNS induced by LPS ICV injection affected the peripheral iron metabolism was unknown, in the present study, we demonstrated the effect of LPS ICV injection on serum iron level and hepcidin expression in the liver, then signaling pathway of IL-6/JAK2/STAT3 was also tested for regulation of hepcidin performed on IL-6 knockout mice (IL-6^{-/-}). In addition, AG490 used as the inhibitor of JAK2 and was further to confirm the activation of IL-6/JAK2/STAT3 pathway respond to LPS ICV injection. Our recent finding will provide the evidence that peripheral liver hepcidin expression and iron metabolism were regulated by CNS infection.

Materials and methods

Mice and Chemicals

Wild type mice (C57BL6, 8-week-old, body weight ~25g) and IL-6 knockout mice (IL6^{-/-}, provided by Jackson Laboratories). The animal care and experimental protocols were performed according to the Animal Management Rules of the Ministry of Health of the People's Republic of China and approved by the Animal Ethics Committees of Fudan University, Shanghai, China. Animal were housed under a standard 12h light-dark cycle with water and food supplied. Animal handling and surgical procedures were carried out in accordance with guidelines on the university animal ethics. The JAK2 inhibitor AG490 was purchased from Selleck chem, the other chemicals were obtained from Sigma (St. Louis, MO, USA)

LPS administration by Intracerebroventricular Injection

LPS by ICV injection was performed as previously described [44]. LPS (*E. coli* 055:B5; 5 µg in 2 µl of sterile saline) or Vehicle (sterile saline, 2 µl) was injected into the cerebral lateral ventricle using 10 µl syringe with a 33 gauge needle, the rate of injection was 0.5 µl/min, stereotaxic positions of ICV injection

were -3.0 mm dorsal/ventral, -1.0 mm lateral, and -0.5mm anterior/posterior from the bregma. After the injection, the needle was kept for an additional 5 mins. tissue samples were harvested for analysis at 6h after LPS ICV injection.

Enzyme-Linked Immunosorbent Assay

The expression of IL-6 protein was investigated by using IL-6 ELISA kits, protocols were according to the supplier's instructions. Briefly, liver tissue was digested and homogenized in RIPA lysis buffer (Beyotime), The tissue lysis solution was centrifuged with 12,000×g at 4°C, after 15mins, the supernatant solution was collected for protein concentration detection and ELISA assay. IL-6 ELISA kits were purchased from R&D systems (Minneapolis, MN, USA).

Real-time PCR (RT-PCR)

Total RNA was extracted using TRIzol reagent (life technology) and cDNA preparation was performed using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher), protocols were accordance with the instructions of the manufacturers. Real-time PCR using fast start universal SYBR Green master and was carried out by specific instrument (LC96, Roche, Switzerland) . Quantitative calculation was based on CT values of each target gene, results were normalized to β -actin. Relative fold of gene expression compared to control was calculated by the $2^{-\Delta\Delta CT}$ method. Primers for IL-6 were provided by Primer Bank. Mouse IL-6: forward, 5'-CTGCAAGAGACTTCCATCCAG-3', reverse, 5'-AGTGGTATAGACAGGTCTGTTGG-3'; mouse hepcidin: forward, 5'-AGAGCTGCAGCCTTTGCAC-3', reverse, 5'-GAAGATGCAGATGGGGAAGT-3'; and reverse, 5'-CAGAGGGGTAGGCTTGTCTC-3'; mouse β -actin: forward, 5'-AAATCGTGCGTGACATCAAAGA-3', reverse, 5'-GCCATCTCCTGCTCGAAGTC-3' [44].

Western Blot

The tissues were homogenized in RIPA lysis buffer and then sonicated on ice as described previously [28] BCA (Pierce, Rockford) method was using to detect the protein concentration. Equivalent amounts of protein (30~40g) were loaded in each sample well. The blots were blocked in skim milk and then incubated with specific primary antibodies overnight at 4°C: anti-pSTAT3 (rabbit polyclonal, 1:1000, Cell Signaling Technology), anti-STAT3 (mouse monoclonal, 1:1000, Cell Signaling Technology), anti-Fpn1 (rabbit polyclonal,

1:1000, Novus), anti-Ferritin-L (rabbit polyclonal, 1:1000, Protein tech), anti- β -actin (mouse monoclonal, 1:10000, Sigma). then the blots were washed in PBS for three times and then incubated with secondary antibody for 2h. (goat anti-rabbit IRDye 800 CW, 1:1000; goat anti-mouse IRDye 800 CW, 1:5000). The intensity of the specific bands was analyzed by the Odyssey image system (Li-Cor, NE, USA).

Serum Iron and Transferrin Saturation Assay

1% pentobarbital sodium was used to anesthetize the mice (40mg/kg body weight, IP injection), blood samples were obtained from abdominal aorta in anticoagulant syringes, commercial kits were using to measure the serum iron and un-saturated iron binding capacity (UIBC) as described [32]. then total iron binding capacity (TIBC, TIBC=serum iron+UIBC) and transferrin saturation (TS, TS = SI/TIBC x 100) were calculated and analyzed.

Statistical Analysis

Graphpad Prism was used to conduct the statistical analyses. Differences between means \pm SD in each two groups were carried out by two-tailed Student's t test, differences between in more than two groups was compared by using One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. All *P* values were two-sided, and *P*<0.05 is considered to be statistically significant.

Results

Hepcidin mRNA, IL-6 mRNA and Protein Expression in the Liver of IL-6^{-/-} Mice was Significantly Lower than IL-6^{+/+} Mice after LPS administration

Based on our previous study showed that hepcidin mRNA in brain was significantly higher in 6h after LPS ICV injection. We first investigated the response of liver in hepcidin and IL-6 expression when mice were ICV injection with LPS. we detected hepcidin mRNA, IL-6 mRNA and protein expression at 6h after LPS ICV injection. The results showed that hepcidin mRNA (Fig. 1A), IL-6 mRNA (Fig.1B) and protein (Fig.1C) significantly increased in the liver of IL-6^{+/+} (wild type) mice with LPS ICV injection. However, these increase was abolished in IL-6 knockout (IL-6^{-/-}) mice, in addition, IL-6^{-/-} mice displayed slightly higher expression of hepcidin mRNA in liver after LPS injection, but there was no significant difference between LPS and PBS-treated IL-6^{-/-} mice (Fig. 1A). these finding indicated that inflammatory reaction in the brain by LPS

ICV injection could affect the hepatic hepcidin mRNA and IL-6 expression.

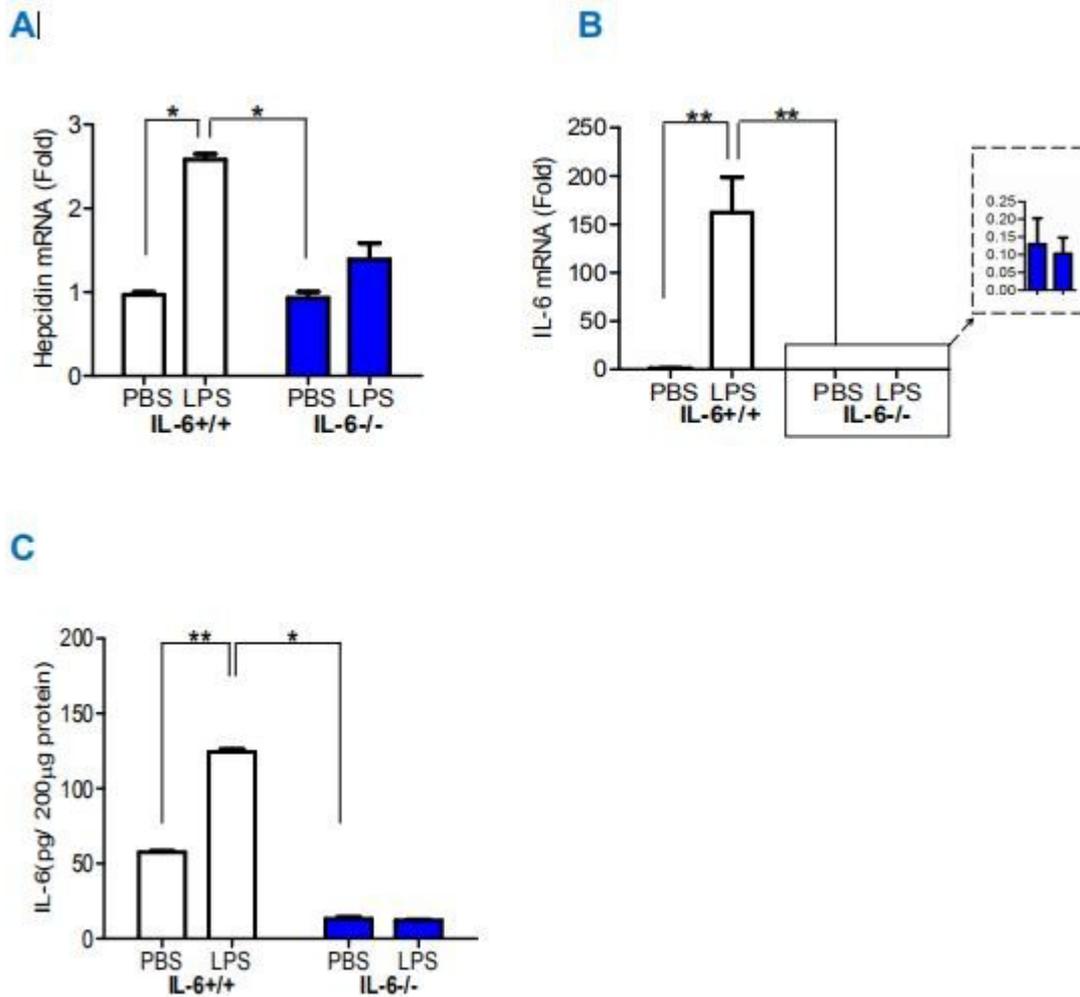


Fig. 1 Expression of hepcidin mRNA, IL-6 mRNA and protein in the liver of LPS-treated IL-6 knockout mice (IL-6^{-/-}) was significantly lower than LPS-treated wild type mice (IL-6^{+/+}). IL-6^{+/+} mice and IL-6^{-/-} mice were ICV injected with LPS (5 μg) for 6h. The expression of hepcidin mRNA (**A**) and IL-6 mRNA (**B**) were detected by Q-PCR, IL-6 protein (**C**) was measured by ELISA. Data represent means ± SEM (% control) (IL-6^{+/+}: PBS = 4, LPS=6; IL-6^{-/-}: PBS = 3, LPS=4). **P* < 0.05; ***P* < 0.01 vs. the mice injected with PBS).

IL-6 Deficiency Abolished the Decrease of Serum Iron and Transferrin Saturation Induced by LPS Injection

We then investigated whether LPS treatment by ICV injection could affect the peripheral iron metabolism, serum Iron and transferrin saturation assay were conducted to confirm the effect of elevated hepcidin expression in the liver of IL-6^{+/+} mice. It was found that serum iron (Fig. 2A) and transferrin saturation

(Fig. 2B) significantly decreased in IL-6^{+/+} mice after LPS treatment, however, the decrease was abolished in IL-6^{-/-} mice. In addition, serum iron and transferrin saturation showed no differences between IL-6^{-/-} mice and IL-6^{+/+} mice after LPS treatment, the serum iron and transferrin saturation in PBS-treated IL-6^{-/-} mice were slightly lower than PBS-treated IL-6^{+/+} mice without significant difference. These results implied that enhanced inflammation in central nervous system could decrease the peripheral serum iron and transferrin saturation.

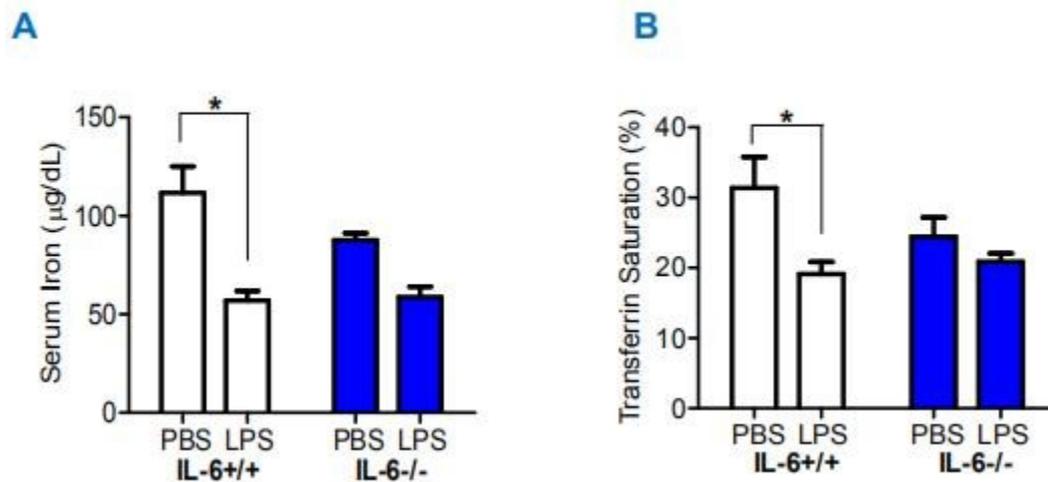


Fig. 2 IL-6 deficiency abolished the LPS induced the reduction of serum iron and transferrin saturation of IL-6^{-/-} mice. IL-6^{+/+} mice and IL6^{-/-} mice were ICV injected with PBS or LPS and sacrificed after 6h. Serum iron concentration (A) and transferrin saturation (B) were measured. Data represent means±SEM (% control) (IL-6^{+/+}: PBS = 4, LPS=6; IL-6^{-/-}: PBS = 3, LPS=4). **P* < 0.05. vs. the mice injected with PBS.

IL-6 Deficiency Abolished the Decrease of Fpn1, Increase of pSTAT3 and Ft-L Protein induced by LPS in Liver

To find out the mechanisms involved in the hepatic hepcidin expression after LPS ICV injection, we then examined phosphorylated STAT3 protein expression in liver, it was found that pSTAT3/STAT3 levels significantly elevated in liver of LPS-treated IL-6^{+/+} mice, however, IL-6 deficiency abolished pSTAT3/STAT3 level in the liver of IL-6^{-/-} mice after LPS treatment (Fig. 3A), these implied LPS ICV injection up-regulated hepcidin expression though IL-6/STAT3 signaling pathway. As previous study showed hepcidin could bind to Fpn1 and induced its internalization [12, 24], and Ft-L protein elevating was a marker of tissue-iron content increasing [33], we also investigated the expression of Fpn1 and Ft-L protein in liver, the results

showed a significant reduction in the expression of Fpn1 protein (Fig. 3B) and an increased expression of Ft-L protein (Fig. 3C) in IL-6^{+/+} mice after LPS treatment, however, such changes were not found in the liver of IL-6^{-/-} mice, in addition, the expression of Fpn1 and Ft-L protein in liver of IL-6^{+/+} mice did not differ from the liver of IL-6^{-/-} mice with PBS treatment. This finding indicated that IL-6 induced by central LPS treatment also participated the iron metabolism in the liver through IL-6/STAT3/hepcidin/Fpn1 axis.

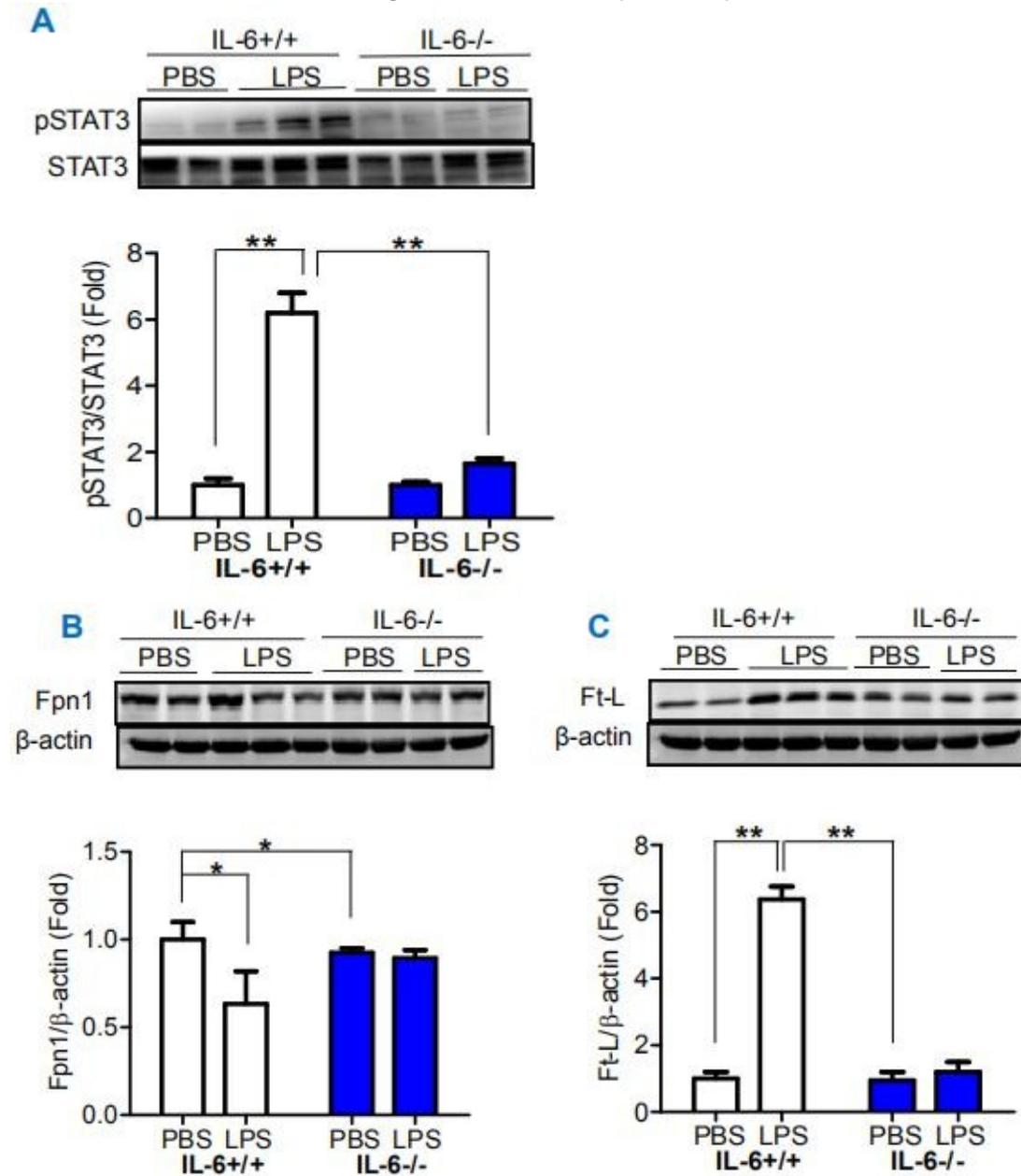
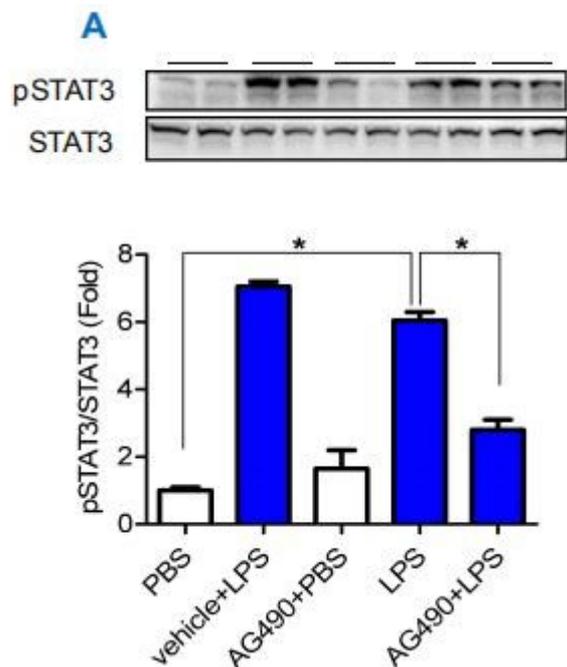


Fig. 3 IL-6 deficiency abolished the LPS induced decrease of Fpn1 and increase of phosphorylation of STAT3 (pSTAT3) and Ft-L protein in the liver of IL-6^{-/-} mice. IL-6^{+/+} mice and IL-6^{-/-} mice were ICV injected with PBS or LPS. At 6h after LPS treatment, pSTAT3 (A), Fpn1 (B) and Ft-L (C) protein

in liver were detected by western blot. Data represent means±SEM (% control) (IL-6+/+: PBS = 3, LPS=6; IL-6-/-: PBS = 3, LPS=3). **P* < 0.05; ***P* < 0.01 vs. the mice injected with PBS.

AG490 Significantly Reduced the pSTAT3 Protein and Abolished the Changes of Fpn1 and Ft-L Expression Induced by LPS in Liver

Finally, we confirmed the role of IL-6/JAK2/STAT3 signaling pathway on hepatic hepcidin expression, AG490 as an inhibitor of JAK2 and IL-6/JAK2/STAT3 pathway was pre-injected (IP injection) into C57BL/6 wild type mice before LPS ICV injection, after pre-treated with AG490, data showed that the ratio level of pSTAT3/STAT3 was dramatically decreased in the liver of mice than LPS-treated alone (Fig. 4A), then, we also investigated the effect of AG490 on Fpn1 and Ft-L protein expression in liver, it was found that inhibiting IL-6/JAK2/STAT3 pathway with AG490 significantly abolished the decrease of Fpn1 (Fig. 4B) and increase of Ft-L protein (Fig. 4C) induced by LPS treatment, This finding confirmed the activation of IL-6/JAK2/STAT3 signaling pathway promoted the hepcidin expression in liver after central LPS treatment.



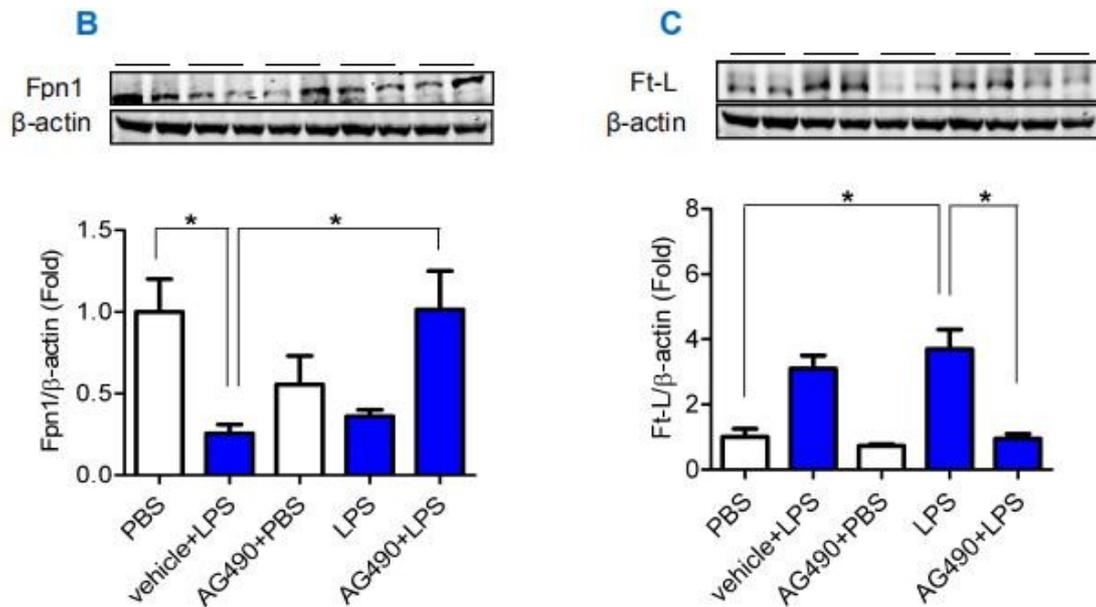


Fig. 4 AG490 significantly reduced the pSTAT3 protein and abolished the changes of Fpn1 and Ft-L protein induced by LPS treatment in liver. C57BL6 wild type male mice were pre-treated with 5mg/kg AG490 (an inhibitor of JAK2) in 10% DMSO (IP injection) or vehicle at 30min before LPS (ICV injection, 5 μ g in 2 μ l sterile saline) for 6h. The expression of pSTAT3 (A), Fpn1 (B) and Ft-L protein (C) in liver were measured by western blot. Data are means \pm SEM (% Control) (n = 4). * $P < 0.05$ vs. the control mice.

Discussion

The major objective of this study was to investigate the effects of CNS inflammation induced by LPS ICV injection on the hepcidin expression and iron metabolism in peripheral liver, and to find out whether the IL-6/JAK2/STAT3 signaling pathway in liver activated the hepcidin expression after LPS injected into brain. In present study, we demonstrated hepcidin and IL-6 mRNA as well as phosphorylation of STAT3 increased significantly after LPS ICV injection, elevated expression of hepcidin led to decreased Fpn1 protein and increased Ft-L protein in liver. However, IL-6 knockout remarkably abolished the hepcidin mRNA expression and suppressed IL-6/JAK2/STAT3 pathway. Our data demonstrated firstly that LPS treatment by ICV injection activated the hepcidin mRNA expression not only in brain, but also in the peripheral liver, it implied that inflammation in CNS also mediated the peripheral iron metabolism.

LPS is the endotoxin from bacteria and primarily triggers inflammation response. Systemic inflammation in animal model is induced with LPS by IP injection or IV injection. However, peripheral animal models with LPS treatment are also widely used to induce neuro-inflammation [35], It has been investigated that macrophages and glia cells, as well as pro-inflammatory cytokines were activated and induced by peripheral treatment of LPS in periphery and brain [37]. Several studies have used LPS by IP or IV injection at variable doses ranging from 0.02 mg/kg to 3 mg/kg body weight. It has been reported that the elevation of hepcidin mRNA expression in liver was about 2 to 3-fold when mice were challenged with IP injection of LPS (0.5 mg/kg or 1µg/g body weight) 4h or 6h later [38, 39]. The increase of hepatic hepcidin and IL-6 mRNA were separately 1.5-fold and 100-fold have been also observed in mice at a signal 2mg/kg dose of LPS by IP injection after 6h [40]. In our present study, a signal dose of total 5µg in 2µl saline of LPS was administered into the cerebral lateral ventricle for 6 hours, our data has showed that hepcidin mRNA expression in liver of IL-6+/+ mice increased up to 2.5-fold compared to control mice with PBS injection, the elevation of IL-6 mRNA in liver was almost 150-fold after LPS ICV injection. These data implied that LPS had no effect on hepcidin mRNA expression when injected to the brain, and it did not differ from the effect of LPS with IP injection at a similar dose and time point. However, LPS is a macromolecule that is unable to cross the BBB to regulate the hepcidin expression in periphery directly[41], we speculated that inflammatory response induced by LPS injection in brain generated auto-amplificatory loops after activation of glia cell in brain, the production of IL-6 and other pro-inflammatory cytokines induced by LPS in brain changed the permeability of BBB and resulted in the leakage of LPS and IL-6 to peripheral circulation. The increase level of IL-6 in circulation also induced the expression of hepatic hepcidin mRNA, however, this hypothesis is worth further investigation.

The increased hepcidin synthesis decreased Fpn1 expression in the liver through binding to and inducing the degradation of Fpn1 in enterocytes of the duodenum and leading to a decrease of serum iron [16]. Therefore, the level of serum iron and transferrin saturation after LPS ICV injection were also investigated. Our data showed that IL-6+/+ mice displayed significant decrease of serum iron and transferrin saturation for LPS treatment after 6h, however, IL-6-/- mice significantly inhibited the decrease of these two indices. While serum iron and transferrin saturation after LPS ICV injection were slightly lower than PBS treatment in IL-6-/- mice with no significant difference.

The results might be due to the partly abolished of hepatic hepcidin expression showed in Fig. 1A, our finding indicated that IL-6 acted the significant role in regulation hepcidin expression and other molecules or inflammatory cytokines were partly involved in hepcidin expression as well [22, 43].

It has been investigated that LPS induced hepcidin expression through IL-6/JAK2/STAT3 signaling pathway [28], We further demonstrated the effect of IL-6/JAK2/STAT3 signaling pathway and phosphorylation of STAT3 in liver, our results showed the significantly increased expression of pSTAT3 protein in liver. As mentioned above, LPS triggered various pro-inflammatory cytokines that induced hepcidin expression, however, our present study was performed on IL-6 knockout mice and mainly focused on the IL-6/JAK2/STAT3 signaling pathway induced by LPS ICV injection. Other mechanisms need to be explored to fully demonstrated the hepatic hepcidin expression with LPS ICV injection. To investigate serum iron reduced by hepcidin, the only known iron exporter Fpn1 and iron storage protein Ft-L were examined, our data showed hepatic Fpn1 expression did not change with PBS treatment in IL-6^{-/-} mice compared to IL-6^{+/+} mice, this changes was consistent with hepcidin mRNA and pSTAT3 protein expression in these two groups, however, our previous study in brain have showed Fpn1 decreased significantly in IL-6^{-/-} mice compared to IL-6^{+/+} mice with PBS treatment [44], based on the different observation in Fpn1 expression, we proposed that the distinct effect of IL-6 on Fpn1 expression in the liver and brain caused the difference, the relevant mechanisms are currently unknown.

AG490 is a compound that inhibits tyrosine kinase and the extensive inhibitor of JAK2/STAT3 signaling pathway, studies in vivo and in vitro have demonstrated that AG490 could significantly inhibit hepcidin expression in hepatocytes and mice model [45, 46]. AG490 exerts inhibitory effect on hepcidin expression through inhibiting the phosphorylation of STAT3 when exposed to IL-6[47]. Therefore, in our present study, AG490 was selected to inhibit IL-6/JAK2/STAT3 signaling pathway and to confirm this pathway participating in the hepcidin expression. our previous study has investigated the effect of stattic on hepcidin expression [44], another direct inhibitor of pSTAT3 expression, the previous study and the present study confirmed that LPS treatment by ICV injection activated the IL-6/JAK2/STAT3 pathway and subsequently induced hepcidin expression in brain and liver.

Conclusion

In summary, we have demonstrated firstly that LPS treatment by ICV injection upregulated hepcidin expression not only in cortex and hippocampus, but also in peripheral liver tissue. Activation of IL-6/JAK2/STAT3 signaling pathway in liver explained why administration of LPS in brain could regulate the hepatic hepcidin expression, however, further studies are needed to define the other molecular mechanisms by which LPS or inflammation up-regulated hepcidin expression in liver. Our data provided further evidence that the effect of hepatic hepcidin expression on peripheral iron metabolism should be considered when central inflammation response induced.

Abbreviations

LPS: Lipopolysaccharide; ICV: Intracerebroventricular; IL-6: Interleukin 6; JAK2: Janus kinase 2; STAT3: Transducer and activator of transcription 3; BBB: Blood Brain Barrier; ROS: reactive oxygen species; HPA: hypothalamicadrenal-pituitary axis; Fpn1: ferroportin; Ft-L: Ferritin light chain; IP: intraperitoneal; IV: intravenous.

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Author's contributions

K.Z. and Z.M.Q. conceived, organized and supervised the study; F.L.Z. conducted ICV injection, real-time PCR and western blot, serum iron and transferrin saturation assay and ELISA. P.Z. analyzed the data. M.K.Z. and Z.M.Q. prepared and revised the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The animal care and experimental protocols were performed according to the Animal Management Rules of the Ministry of Health of the People's Republic of China, and approved by the Animal Ethics Committees of Fudan University, Shanghai, China. Animal handling and surgical procedures were carried out in accordance with guidelines on the university animal ethics.

Consent for publication

Not applicable

Competing interests

The authors declare no competing financial interests

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