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Effects of IL-10 on iron metabolism in LPS-induced inflammatory mice via modulating hepcidin expression

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Abstract. – OBJECTIVE: Body's iron metabolism is at one dynamic balance status, and abnormal iron metabolism may lead to renal anemia. Inflammation stimuli may lead to abnormal iron metabolism and aggravation of chronic failure anemia. Hepcidin can regulate iron metabolic homeostasis, further mediating renal anemia. Interleukin-10 (IL-10) is an inflammatory inhibitor, but with an unclear function in the regulation of hepcidin expression.

MATERIALS AND METHODS: BALB/c mice were randomly assigned into three groups: control group; lipid polysaccharide (LPS) group, which received 0.1 mg/kg LPS via tail veins; IL-10 group with 0.2 mg/kg IL-10 injection after LPS. Red blood cell count (RBC), hemoglobulin (Hb), hematocrit (HCT), mean corpuscular volume (MCV) and iron content in hemoglobulin were measured. Real-time PCR quantified hepcidin mRNA expression in all groups. Enzyme linked immunosorbent assay (ELISA) tested serum hepcidin, IL-6 and tumor necrosis factor-α (TNF-α) levels. Western blot analyzed expression of mouse transferrin receptor 2 (TfR2) and hepcidin signal pathway molecule STAT3.

RESULTS: LPS model group had lower RBC, Hb, HCT, MCV and iron content in Hb, plus elevated hepcidin, IL-6, TNF-α, TfR2 and STAT3 expression ($p < 0.05$ compared to the control group). IL-10 treatment group significantly facilitated RBC, Hb, HCT, MCV and Hb iron contents in LPS-induced inflammatory model mice, which also had lower hepcidin, IL-6, TNF-α, TfR2 or STAT3 expression ($p < 0.05$ compared to LPS group).

CONCLUSIONS: IL-10 can improve iron metabolism and alleviate anemia via suppressing inflammatory factor, modulating STAT3 signal pathway, down-regulating hepcidin expression and inhibiting TfR expression.

Key Words:
IL-10, hepcidin, TfR2, Iron metabolism, Anemia.

Introduction

As a necessary trace element, iron participates in various body growth and development processes, including hemoglobulin (Hb) for blood oxygen transportation, synthesis of myoglobin for energy metabolism, DNA synthesis and metabolism, immune cell proliferation and differentiation[1,2]. Excess or deficiency of iron both lead to body diseases such as renal anemia and hemochromatosis, further damaging body tissue/organs[3]. Therefore, the regulation of iron metabolism is of critical importance for maintaining homeostasis[4]. Chronic renal failure (CRF) patients are often complicated with anemia, which severely affects patient life quality, or even elevates mortality[5]. Inflammatory stimulus is closely correlated with anemia pathogenesis, and can lead to iron metabolic disorder and aggravate anemia, thus playing a regulatory role in CRF-related renal anemia[6,7]. Therefore, the regulation of iron metabolic balance plays a crucial role in alleviating renal anemia. Hepcidin, or named as liver-expressed antimicrobial peptide, has been shown to paly critical roles in maintaining dynamic homeostasis of iron metabolism[8]. Hepcidin is mainly produced and secreted by hepatocytes, and is excreted in urine. Bioactive peptide hepcidin is lysed from prohepcidin by proteinase, and regulates duodenal absorption of iron, plus tissue release of iron elements, thus affecting iron metabolic homeostasis via modulating tissue absorption and secretion of iron[9,10]. Internal stimuli such as inflammation, hypoxia and endoplasmic reticulum stress can lead to elevated expression of hepcidin mRNA and protein, further aggravating iron metabolic disorder and anemia[11,12]. IL-10 is one pluripotent negative regulatory, and is mainly produced by Th2 cells, activated B cells, monocytes, and

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macrophage[13,14]. IL-10 participates in biological regulation of immune cells, inflammatory cells, and tumor cells, thus playing important roles in autoimmune disease, severe infectious disease, tumor, and transplant immunity[15,16]. Whether IL-10 can affect iron metabolism via modulating hepcidin has not been reported.

Materials and Methods

Experimental Animals

Healthy male BALB/c mice (2 months age, specific pathogen free (SPF) grade, body weight 22 ±5 g) were purchased from Laboratory Animal Center of our institute and were kept in an SPF grade animal facility with fixed temperature (21 ± 1°C) and fixed humidity (50-70%) with 12 h light/dark cycle. Mice were used for all experiments and all procedures were approved by the Animal Ethics Committee of Affiliated Hospital of Youjiang Medical College for Nationalities.

Major Reagents and Equipment

10% hydrate chloral was purchased from Zhaohui Pharm (Shanghai, China). IL-10 and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) was purchased from Pall Life Sciences (Covina, CA, USA). Western blot reagents were purchased from Beyotime Biotechnology (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-mouse TfR2 monoclonal antibody, rabbit anti-mouse signal transducer and activator of transcription 3 (STAT3) monoclonal antibody, and goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Enzyme linked immunosorbent assay (ELISA) kits for hepcidin, TNF-α and IL-6 were purchased from R&D Systems Inc. (Minneapollis, MN, USA). Surgical microscope instruments were purchased from Suzhou Medical Instrument (Suzhou, China). AU5800 fully automatic biochemical analyzer was purchased from Beckman Coulter (München, German). Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Other common reagents were purchased from Sangon Biotech. (Shanghai, China).

Animal Grouping and Treatment

BALB/c mice were randomly assigned into three groups: control group; LPS group, which received 0.1 mg/kg LPS via tail veins; IL-10 group, which received 0.2 mg/kg IL-10 via tail vein injection after inflammatory model preparation. All animal protocols followed Ethical Guidelines.

Sample Collection

After injecting LPS or IL-10 for 6 h, mice were anesthetized by 10% hydrate chloral. Blood samples were collected from abdominal aorta using vacuum tubes. After room temperature incubation for 30 min, the blood sample was centrifuged at 4°C for 10 min at 3600 r/min. The supernatant was saved at -20°C for further usage. Mice after sacrifice were collected for right hepatic lobules, which were frozen at -80°C for storage.

Mouse Blood Index Assay

A fully automatic biochemical analyzer was used to analyze RBC, Hb, HCT, MCV and Hb iron contents of all groups of mice.

Real-time PCR for Hepcidin mRNA Expression in Mouse Liver Tissues

Trizol reagent was used to extract hepatic tissue mRNA. DNA reverse transcription was performed following the instruction of test kit. Primers were designed based on target gene sequence using PrimerPremier 6.0, and were synthesized by Invitrogen (Carlsbad, CA, USA) as Table I. Real-time PCR was performed for measuring target gene expression under following conditions: 35 cycles each containing 92°C 30 s, 58°C 45 s and 72°C 35 s. Data were collected and calculated for CT values of all standards and samples based on fluorescent quantification and using GAPDH as the internal reference. Standard curve was plotted for semi-quantitative analysis by $2^{-\Delta Ct}$ method.

Table I. Primer sequences.

Gene	Forward primer 5′-3′	Reverse primer 5′-3′
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
Hepcidin	CAGGTACTACCTACCGTAGT	ATTTCACTTCTTTCACTCACA

ELISA for Expression of Serum Inflammatory Factors Hepcidin, TNF-α and IL-1β

Serum from all groups was collected and was measured for TNF-α and IL-1β expression following manual instruction of ELISA kit. In brief, 96-well plate was added with 50 μl serially diluted samples, which were used to plot standard curves. 50 μl test samples were then added to test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 s vortex. The rinsing procedure was repeated for 5 times. A total of 50 μl enzyme labeling reagent was then added to each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 μl each), followed by 37°C dark incubation for 10 min. The test plate was then mixed with 50 μl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. Linear regression model was then plotted based on the concentration of standard samples and respective A values. Sample concentration was further deduced based on optical density (OD) values using the regression function.

Western Blot for Protein Expression of TfR2 and STAT3

Total protein samples were firstly extracted from hepatic tissues. In brief, radio-immunoprecipitation assay (RIPA) lysis buffer was used to lyse cells on ice for 15-30 min, followed by ultrasound rupture (5 s × 4) and centrifugation (4°C, 10000 × g, 15 min). Supernatants were saved and quantified for protein contents by Bradford method, and were stored at -20°C for further Western blotting. Proteins were then separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and were transferred to polyvinylidene fluoride (PVDF) membrane using semi-dry method (100 mA, 1.5 h). Non-specific background was removed by 5% defatted milk powder at room temperature for 2 h, followed by the addition of anti-TfR2 monoclonal antibody (1:1000) or anti-STAT3 monoclonal antibody (1:500) in 4°C overnight incubation. On the next day, the membrane was rinsed in PBST and was incubated with 1: 2000 goat anti-rabbit secondary antibody for 30 min. After PBST rinsing, ECL reagent was used to develop the membrane for 1 min, followed by exposure under X-ray for observation. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated for four times (n=4) for further analysis.

Statistical Analysis

SPSS16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data were presented as mean ± standard deviation (SD). Comparison of means among multiple groups was performed by one-way analysis of variance (ANOVA), followed Dunnett post hoc test. A statistical significance was defined when $p < 0.05$.

Results

Blood Index of all Groups of Mice

In LPS-induced inflammatory mice, blood indexes were changed, as shown by lower RBC, Hb, HCT or MCV ($p < 0.05$ compared to control group). IL-10 treatment on inflammatory model increased RBC, Hb, HCT and MCV levels ($p < 0.05$ compared to model group, Table II).

Effects of IL-10 on Serum Iron Content in Inflammatory Mice

LPS-induced inflammatory mice had decreased serum iron level ($p < 0.05$ compared to control group). IL-10 treatment significantly increased serum iron contents ($p < 0.05$ compared to model group, Figure 1).

Table II. Blood index change of mice.

Index	Control	Model	Treatment
RBC (10^{12}/l)	8.3 ± 1.7	5.2 ± 2.1*	7.6 ± 1.2#
Hb (g/dl)	13.4 ± 2.2	9.1 ± 1.3*	11.2 ± 1.5#
HCT	0.5 ± 0.1	0.2 ± 0.1*	0.4 ± 0.21#
MCV (fg)	50.3 ± 4.3	31.5 ± 2.2*	43.8 ± 3.6#

Note: *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

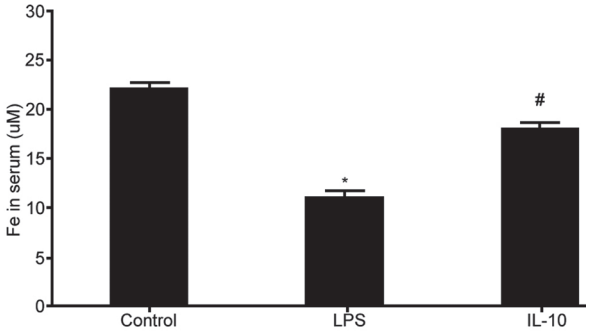

Figure 1. IL-10 effects on serum iron content in inflammatory mice. *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

Effects of IL-10 on Hepcidin Expression in Inflammatory Mice

Real-time PCR and ELISA were used to test the effect of IL-10 on mRNA and serum expression of hepcidin in inflammatory mice. Results showed that LPS-induced inflammatory mice had elevated hepcidin mRNA expression in hepatic tissues, plus higher serum hepcidin expression ($p < 0.05$ compared to control group). IL-10 treatment on inflammatory model decreased mRNA and serum hepcidin expression ($p < 0.05$ compared to model group, Figure 2).

Effects of IL-10 on Serum Inflammatory Factors Expression in Inflammatory Mice

ELISA was used to analyze differential expression of serum inflammatory factors including TNF-α and IL-1β. Results showed significantly elevated serum inflammatory factors TNF-α and IL-1β in model group ($p < 0.05$ compared to control group). IL-10 treatment significantly suppressed TNF-α and IL-1β expression ($p < 0.05$ compared to model group, Figure 3).

Effects of IL-10 on TfR2 Expression in Inflammatory Mice

Western blot was employed to test the effect of IL-10 on TfR2 expression in inflammatory mice. Model group had elevated TfR2 protein expression ($p < 0.05$ compared to control group). IL-10 treatment significantly inhibited TfR2 expression ($p < 0.05$ compared to model group, Figure 4).

Effects of IL-10 on STAT3 Expression in Inflammatory Mice

Western blot was used to test the effect of IL-10 on STAT3 expression in inflammatory mice. Model group had elevated STAT3 protein expression in mouse liver tissues ($p < 0.05$ compared to control group). IL-10 treatment remarkably inhibited STAT3 expression ($p < 0.05$ compared to model group, Figure 5).

Discussion

LPS is composed of core polysaccharide, O antigen and lipid-like A component, and is the major constitute of Gram-negative bacterial cell wall endo-toxin[17]. LPS can activate body macrophage, monocyte and endothelial cells, for the release of inflammatory factors to cause systemic inflammatory response[18]. A previous work showed that inflammatory stimulus can cause iron-deficient environment for causing anemia[19]. Major iron regulatory hormone hepcidin, or named as iron regulator, is an important mediator for inflamma-

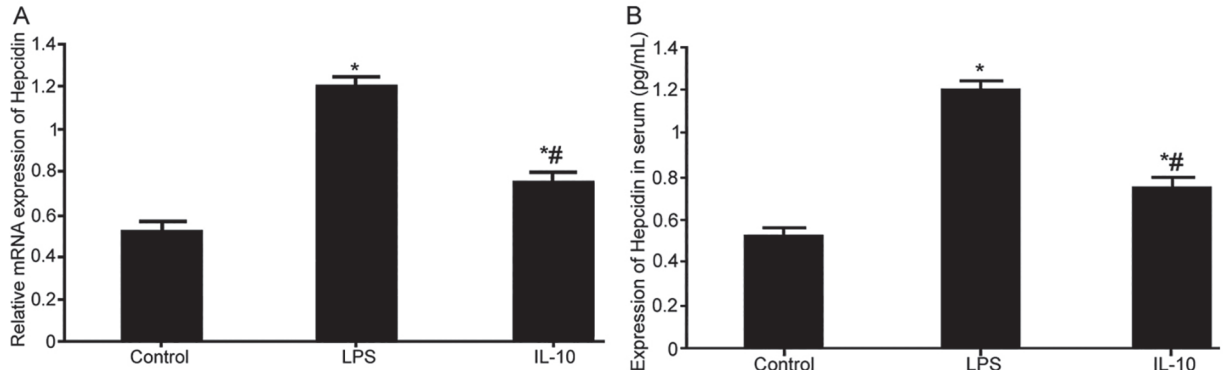

Figure 2. Effects of IL-10 on hepcidin expression in inflammatory mice. ***A,*** Real-time PCR for the effect of IL-10 on Hepcidin mRNA expression in inflammatory mice. ***B,*** ELISA for IL-10 effects on serum Hepcidin expression in inflammatory mice. *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

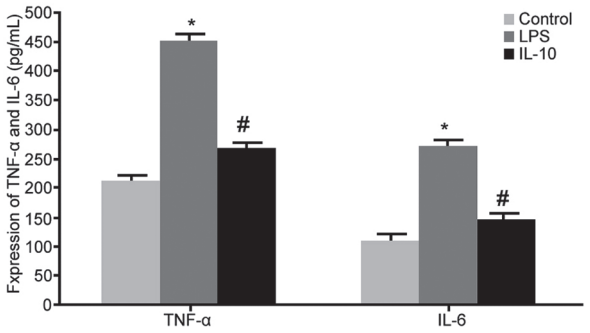

Figure 3. Effects of IL-10 on inflammatory factor expression in mouse serum.*, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

tory anemia[20]. This study utilized LPS-induced mouse inflammatory model, on which blood indexes were changed, with decrease of RBC, Hb, HCT and MCV, leading to anemia symptom and lower serum iron level. On the other hand, hepcidin mRNA and serum expressions are enhanced, as consistent with previous reports showing pathologically increased hepcidin under iron deficient anemia or inflammatory disease[21]. Chronic infection and inflammation activate body defense mechanism to prevent microbial utilization of iron. Therefore, intestinal iron absorption can be decreased by facilitating hepcidin expression, whilst macrophage cannot timely release iron to

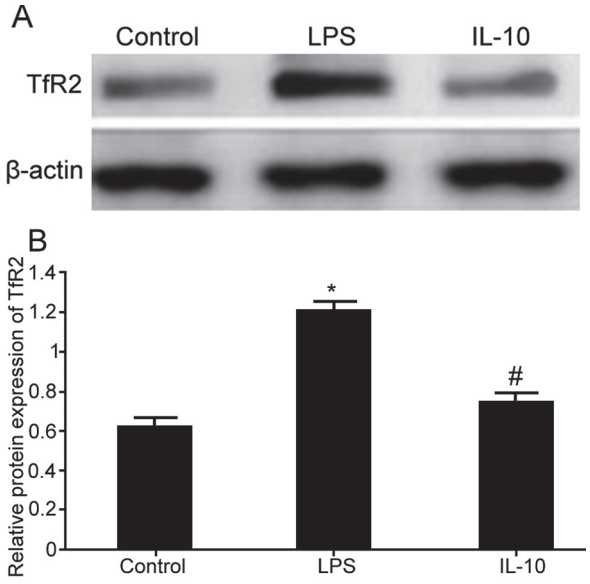

Figure 4. Effects of IL-10 on TfR2 expression in inflammatory mice. ***A,*** Western blot for the effect of IL-10 on TfR2 expression in inflammatory mice. ***B,*** IL-10 effects on TfR2 expression in inflammatory mice. *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

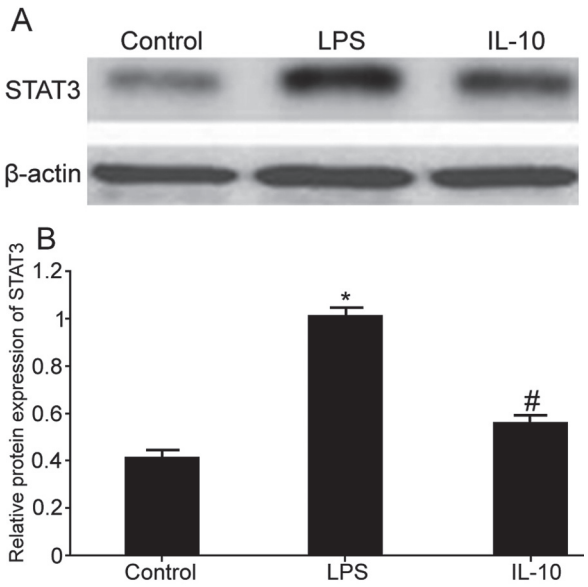

Figure 5. Effects of IL-10 on STAT3 expression in inflammatory mice. ***A,*** Western blot for the effect of IL-10 on STAT3 expression in inflammatory mice. ***B,*** IL-10 effects on STAT3 expression in inflammatory mice. *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

cause iron deficient anemia[22]. Further analysis of IL-10 effects and mechanisms in inflammatory iron metabolism demonstrated that IL-10 significantly facilitated RBC, Hb, HCT, MCV and Hb-iron contents in LPS inflammatory model mice, which also had decreased hepcidin mRNA/protein expression, IL-6 or TNF-α levels, and lower TfR2 or STAT3 expression. As one anti-inflammatory factor, IL-10 can suppress inflammatory response and antagonize the effect of inflammatory mediator[23]. Inflammatory factors IL-6 and TNF-α can facilitate hepcidin expression for mediating iron metabolic homeostasis via up-regulating hepcidin signal pathway STAT3 and leading to increased expression of TfR2[24]. IL-10 can down-regulate hepcidin expression via inhibiting IL-6 and TNF-α inflammatory factors and further suppressing STAT3 expression, therefore improving iron metabolic homeostasis and inflammatory anemia symptoms.

Conclusions

IL-10 can improve iron metabolism and regulate anemia via suppressing inflammatory factors to mediate STAT3 signal pathway, thus suppressing hepcidin mRNA or protein expression, further suppressing TfR expression.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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