The effect of anti-inflammatory properties of ferritin light chain on lipopolysaccharide-induced inflammatory response in murine macrophages

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Ferritin light chain (FTL) reduces the free iron concentration by forming ferritin complexes with ferritin heavy chain (FTH). Thus, FTL competes with the Fenton reaction by acting as an antioxidant. In the present study, we determined that FTL influences the lipopolysaccharide (LPS)-induced inflammatory response. FTL protein expression was regulated by LPS stimulation in RAW264.7 cells. To investigate the role of FTL in LPS-activated murine macrophages, we established stable FTL-expressing cells and used shRNA to silence FTL expression in RAW264.7 cells. Overexpression of FTL significantly decreased the LPS-induced production of tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), nitric oxide (NO) and prostaglandin E2 (PGE2). Additionally, overexpression of FTL decreased the LPS-induced increase of the intracellular labile iron pool (LIP) and reactive oxygen species (ROS). Moreover, FTL overexpression suppressed the LPS-induced activation of MAPKs and nuclear factor-κB (NF-κB). In contrast, knockdown of FTL by shRNA showed the reverse effects. Therefore, our results indicate that FTL plays an anti-inflammatory role in response to LPS in murine macrophages and may have therapeutic potential for treating inflammatory diseases.

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1. Introduction

Ferritin, an iron storage molecule, is mainly localized in the cytoplasm, trace amounts are also detected in the serum and other biological fluids [1]. The cytoplasmic form of mammalian ferritin consists of two subunits: ferritin heavy chain (FTH) and ferritin light chain (FTL). FTH has high ferroxidase activity and converts Fe (II) to Fe (III), and FTL, ferritin light chain; LPS, lipopolysaccharide or endotoxin; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; ERK, extracellular-signal-related kinase; NF-κB, nuclear factor-κB; TR1, transferrin receptor 1; LIP, labile iron pool; ROS, reactive oxidative species; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; SIH, salicylaldehyde isonicotinoyl hydrazone.

Abbreviations: FTL, ferritin light chain; LPS, lipopolysaccharide or endotoxin; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; ERK, extracellular-signal-related kinase; NF-κB, nuclear factor-κB; TR1, transferrin receptor 1; LIP, labile iron pool; ROS, reactive oxidative species; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; SIH, salicylaldehyde isonicotinoyl hydrazone.

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decreasing inflammatory reactions, which are the main cause of inflammatory diseases. Previous reports showed that MAPKs and NF-κB signaling pathways are closely related to the LPS-induced synthesis of TNF-α and IL-1β in macrophages. These signaling pathways are also involved in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in macrophages, which catalyze the production of NO and PGE2, respectively [12-15]. LPS-induced reactive oxygen species (ROS) upregulation is a potent inducer of MAPKs and NF-κB signaling pathways [16]. Free iron, which is found in the cellular labile iron pool (LIP), acts as a catalytic agent for Fenton reactions and participates in the generation of free radicals that possess unpaired electrons such as HO•. Free radicals are generally known as ROS and result in oxidative damage [17].

We hypothesized that FTL may exert an anti-inflammatory role in the LPS-induced inflammatory response that involves inhibiting LIP upregulation and ROS production. In the current study, we developed a RAW264.7 murine macrophage-based cell line that either stably overexpressed the FTL protein or was depleted of FTL through shRNA technology. These cells were used to clarify the role of FTL in the LPS-induced inflammatory response in activated macrophages. Our results demonstrated the role for FTL in LPS-induced activation of signaling pathways and the development of the inflammatory process. Our findings suggest that FTL exerts an anti-inflammatory role in LPS-induced inflammatory response, and modulation of FTL may be a potential approach for anti-inflammatory therapy.

2. Materials and methods

2.1. DNA constructs

The pcDNA3-Flag-FTL (pFTL) construct was generated by inserting the mouse FTL cDNA (NCBI accession number: NM_010240) into pcDNA3 (Invitrogen, Carlsbad, CA, USA). The Flag epitope tag was introduced at the 3′ end of the coding sequence of the gene. The FTL shRNA expression vector, pRNA-U6.1/neo-shFTL (shFTL), was constructed using pRNA-U6.1/neo (GenScript, Piscataway, NJ, USA). To generate an expression vector, pRNA-U6.1/neo-shFTL (shFTL), was constructed by inserting the resultant double-stranded DNA fragments into the BamHI-HindIII sites of pRNA-U6.1/neo. All of the constructs were demonstrated the role for FTL in LPS-induced activation of signaling pathways and the development of the inflammatory process. Our findings suggest that FTL exerts an anti-inflammatory role in LPS-induced inflammatory response, and modulation of FTL may be a potential approach for anti-inflammatory therapy.

2.2. Cell culture and stable transfection

RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People’s Republic of China). RAW264.7 cell clones stably expressing pFTL or shFTL were constructed by transfecting the cells with pFTL or shFTL using lipofectamine™ 2000. Cells were grown in Dulbecco’s modified Eagle’s medium (GBICO, Grand Island, NY) with 10% fetal bovine serum, G418 (600 μg/ml), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO2. The transfected cells were cultured in the presence of G418, which was gradually increased to 800 μg/ml. After a period of 4–5 weeks, the G418-resistant colonies were picked. Corresponding empty vector clones were used as controls. These clones were then expanded and screened for the following experiments.

2.3. Antibodies and reagents

Antibodies for FTL, ferritin, TfR1 and NF-κB (p65 subunit) were purchased from Abcam (Hong Kong, China). Antibodies for p38, p44/42 (ERK1/2), β-Box, p-p44/42 (p-ERK1/2) (Thr202/Tyr204), p-p38 (Thr180/Tyr182) and p-β-Box (Ser32/36) were obtained from Cell Signaling Technology (Danvers, MA, USA). The polyclonal antibody for iNOS was obtained from Cayman (Ann Arbor, MI, USA). The polyclonal antibody for COX-2 was obtained from Abcam (Burlingame, CA, USA). All secondary antibodies used for western blotting were purchased from Cell Signaling Technology. The Flag-tag antibody, LPS, calcine-AM and DCFH-DA were purchased from Sigma. G418 was from Amresco.

2.4. Reverse transcription-PCR analysis

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. A reverse transcription reaction of 2 μg of each total RNA was performed at 42 °C for 1 h. PCR was performed using the Mastercycler Gradient RT-PCR System (Eppendorf) with the following primers: TNF-α: sense 5′-agcacagaaagtga-3′, antisense 5′-cagagaagtttaacctcc-3′; IL-1β: sense 5′-aagctttctgcccctcctt-3′, antisense 5′-gagcttgacacgctctt-3′; COX-2: sense 5′-ctctcaccttctc-3′, antisense 5′-gcagagtgttctgacat-3′; β-actin: sense 5′-agacctgtaacggctacc-3′, antisense 5′-tggattgcacggattt-3′. PCR products were resolved on 1.2% agarose gels and stained with ethidium bromide. β-actin was used as a loading control where indicated.

2.5. Western blot

Cells were rinsed twice with ice-cold PBS and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF) membranes for 30 min. Cell lysates were centrifuged at 15,000 × g at 4 °C for 15 min [9]. The supernatant was collected, and the protein concentration was measured with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Aliquots of the extract containing 50 μg of protein were denatured in SDS and subjected to SDS–PAGE followed by transfer onto polyvinylidene difluoride (PVDF) membranes for 90 min at RT. The membranes were blocked with blocking buffer containing 5% non-fat milk for 1.5 h at room temperature and then incubated with primary antibody overnight at 4 °C. The antibody–antigen complexes were visualized using Super Signal West Pico (Pierce). The total density of the protein bands was detected with the LAS4000 System (FujiFilm).

2.6. ELISA assay

RAW264.7 cells were seeded in 24-well plates at 2.5 × 104 cells/well 24 h before the experiments. After LPS treatment, media were collected and centrifuged at 10,000 rpm for 5 min. The contents of TNF-α, IL-1β and PGE2 were determined by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using the mouse ELISA kits (Roche) for TNF-α and IL-1β and PGE2 (Cayman) according to the manufacturers’ instructions.

2.7. Nitrite analysis

RAW264.7 cells were seeded in 24-well plates at 2.5 × 105 cells/well 24 h before the experiment. Cells were treated with LPS (100 ng/ml) for 12 h. NO synthesis was then determined by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid) as described in [9]. Absorbance was measured at 550 nm.

2.8. Immunofluorescence assay

For the detection of the intracellular localization of the NF-κB p65 subunit, cells were grown on glass coverslips at a seeding density of 2 × 104 cells/ml. After LPS (100 ng/ml) treatment for 30 min, the
cells were fixed in 4% paraformaldehyde at room temperature, and then the membrane was permeabilized in 0.2% Triton X-100 in cold PBS for 30 min. Non-specific binding sites were blocked with 5% bovine serum albumin (BSA) in PBS (0.2% Tween-20 in PBS) at room temperature for 30 min. The rabbit anti-NF-κB p65 subunit (1:300) primary antibody was then incubated with the cells for 1 h at room temperature. After sufficient washes with PBS, cells were incubated with FITC-labeled goat anti-rabbit IgG antibody (1:500) for 1 h at room temperature in the dark and then washed with PBS for 10 min. Cells were stained with DAPI (1 μg/ml) for 10 min at 37 °C in the dark followed by sufficient washes with PBS. Stained cells were analyzed on the Axio Imager A2 microscope (Carl Zeiss) using an excitation wavelength of 490 nm and an emission wavelength of 540 nm for FITC and an excitation wavelength of 360 nm and an emission wavelength of 450 nm for DAPI.

2.9. Labile iron pool measurements

The LIP of the cells was assessed by the calcein-based method. Briefly, cells were loaded with 100 nM calcein-AM for 30 min at 37 °C after LPS (100 ng/ml) treatment for 30 min and then washed with PBS three times to remove free dye. Baseline calcein fluorescence was measured on a fluorescence plate reader (excitation 488 nm, emission 517 nm). The amount of intracellular iron bound to calcein was assessed by addition of 100 μM of the specific iron chelator SIH (salicylaldehyde isonicotinoyl hydrazone; a gift from Prof. P. Ponka, Lady Davis Institute for Medical Research, Montreal, Canada) for 30 min followed by measurement of the fluorescence signal. Triplicate wells were used for each condition. Cell viability (assayed by Trypan Blue dye exclusion) was >95% and unchanged during the assay.

2.10. Measurement of intracellular ROS levels

2′,7′-Dichlorofluorescein-diacetate (DCFH-DA) is a well-established compound for detecting and quantifying intracellular ROS [18]. Intracellular ROS was measured by detecting the fluorescence intensity of DCF through the DCFH-DA→DCF→DCFH conversion as previously described [19]. Briefly, after washing with PBS three times, cells were incubated with DCFH-DA (1 μM) at 37 °C for 30 min in the dark and then treated with PBS containing 100 ng/ml LPS for 30 min. The fluorescence was detected immediately by flow cytometry at wavelengths of 488 nm for excitation and 517 nm for emission (FACSCalibur, BD Biosciences). DCF fluorescence images and cell morphology images were examined using an Olympus fluorescence microscope (Tokyo, Japan). An increased value compared to control was viewed as the increase of intracellular ROS.

2.11. Measurement of exogenous Flag-FTL iron content

Exogenous Flag-FTL iron content was measured according to the literature [20]. For the present study, lysates from pcDNA3 stable expression cells and pFTL stable expression cells were used for immunoprecipitation (IP) experiments with a Flag antibody.

2.12. Statistical analysis

Statistical analysis used the program SPSS 12.0 (SPSS, Chicago, IL, USA). Results are expressed as means ± SEM. Differences between means are determined by one-way ANOVA followed by Student–Newman–Keuls tests for multiple comparisons and Student’s t test for other data. A value of p < 0.05 was considered statistically significant.

3. Results

3.1. LPS induces FTL expression in RAW264.7 cells

An earlier report showed that the lung ferritin content increased significantly after the introduction of LPS into the lungs of rats [21]. However, studies of FTL upregulation in LPS-stimulated macrophages have not been reported. In the present study, LPS expression in LPS-stimulated RAW264.7 macrophage-like cells was first assessed by western blot analysis. The RAW264.7 cells were treated with LPS (100 ng/ml) for 0–24 h. As shown in Fig. 1A, FTL protein expression was downregulated within the first 4 h and upregulated after 6 h of LPS treatment. These results suggest that FTL may play a regulatory role in inflammatory response to LPS.

To investigate the physiological role of FTL during the LPS-induced inflammatory response, we constructed a FTL shRNA (shFTL) construct and a Flag-tagged expression plasmid (pFTL). We also established stable
FTL-overexpressing and FTL-knockdown RAW264.7 cell clones by transfecting pFTL and shFTL into RAW264.7 cells, and subsequently selecting with G418 (Fig. 1B, C).

3.2. FTL inhibits LPS-induced production of TNF-α and IL-1β in RAW264.7 cells

TNF-α and IL-1β, the most important inflammatory products, are primarily involved in promoting the LPS-induced inflammatory response. To evaluate the effect of FTL expression on the production of pro-inflammatory cytokines, pFTL and shFTL RAW264.7 clones were treated with LPS (100 ng/ml) for 12 h, and the mRNA levels of TNF-α and IL-1β were determined by RT-PCR. We found that the LPS-induced increase in TNF-α and IL-1β mRNA was inhibited in pFTL clones (Fig. 2A). Further ELISA experiments showed that LPS-induced secretion of TNF-α and IL-1β increased more than 20-fold after 12 h incubation in the pFTL clones compared with the untreated control cells. In contrast, there were less TNF-α (Fig. 2B) and IL-1β (Fig. 2C) released from pFTL cells. Knockdown of FTL by shFTL enhanced the LPS-induced TNF-α and IL-1β mRNA expression and secretion (Fig. 2D–F). These data demonstrate that FTL plays an anti-inflammatory role during the LPS-induced inflammatory response.

3.3. FTL inhibits LPS-induced production of NO and PGE2

NO and PGE2 are critical inflammatory mediators that are continuously produced by iNOS and COX-2 in macrophages in the presence of LPS [22]. To confirm the anti-inflammatory function of FTL, we tested the effect of FTL on mRNA and protein expression of iNOS and COX-2. As shown in Fig. 3, upon exposure to LPS (100 ng/ml) for 12 h, iNOS mRNA and protein expression increased more than 2.5-fold and COX-2 expression increased 7-fold compared with untreated cells. Overexpression of FTL in pFTL clones significantly inhibited the LPS-induced mRNA (Fig. 3A) and protein expression (Fig. 3B) of iNOS and COX-2. To confirm these effects, we measured the secretion of NO and PGE2 in RAW264.7 cells. As shown in Fig. 3C and D, the LPS-induced secretion of NO and PGE2 increased dramatically after 12 h incubation compared with untreated control cells. Additionally, there were less NO and PGE2...
Fig. 3. FTL inhibits LPS-induced production of NO and PGE2. (A–D) The pFTL and pcDNA3 RAW264.7 clones were treated with or without LPS (100 ng/ml) for 12 h. The mRNA levels of iNOS and COX-2 were measured by RT-PCR (A). β-actin was used as a control. The protein levels of iNOS and COX-2 were determined by western blot analysis (B). β-actin was used to verify equal loading in each lane. The culture media were collected for NO (C) and PGE2 (D) assays as described in the Materials and methods. $p < 0.001$, $p < 0.01$, compared with untreated pcDNA3 cells; $p < 0.01$, $p < 0.001$, compared with LPS-treated pFTL cells. (E–H) The shFTL and pRNA-U6.1/neo RAW264.7 clones were treated with or without LPS (100 ng/ml) for 12 h. The mRNA levels of iNOS and COX-2 were measured by RT-PCR (E). The culture media were collected for NO (G) and PGE2 (H) assay. $p < 0.001$, $p < 0.01$, compared with untreated pRNA-U6.1/neo cells; $p < 0.01$, $p < 0.001$, compared with LPS-treated shFTL cells. The experiments were repeated three times and similar results were obtained. The figure shows a representative result.
LIP was increased 2.5-fold compared with untreated cells. The LPS-measured LIP levels using calcein. LIP is involved in the LPS-induced activation of MAPKs and NF-κB and participates in the generation of ROS\[23,24\]. To investigate whether free iron, known as LIP, acts as a catalytic agent for Fenton reactions, we analyzed the role of FTL in the regulation of the mediators of inflammatory response such as septic shock and SIRS that have high mortality. Here, we investigated whether FTL could inhibit the nuclear translocation of NF-κB p65 in LPS-treated RAW264.7 cells.}

3.4. FTL suppresses the LPS-induced increase of intracellular LIP and ROS in RAW264.7 cells

ROS contributes to the LPS-induced activation of MAPKs, as well as NF-κB, and induces the pathogenesis of diverse inflammatory diseases. Free iron, known as LIP, acts as a catalytic agent for Fenton reactions and participates in the generation of ROS\[23,24\]. To investigate whether LIP is involved in the LPS-induced activation of MAPKs and NF-κB, we measured LIP levels using calcein fluorescence as described in the Materials and methods. We treated cells directly with LPS (100 ng/ml) for 30 min and determined the intracellular LIP level. Notably, intracellular LIP was increased 2.5-fold compared with untreated cells. The LPS-induced LIP increase was inhibited in pFTL transfected cells (Fig. 4A). In contrast, the LIP level was higher in shFTL transfected cells than in LPS-treated control cells (Fig. 4A). These results indicate that FTL plays an important role in the LPS-induced LIP upregulation and may have a role in LPS-induced ROS accumulation. Furthermore, we examined the effect of overexpression of exogenous FTL on ROS production. The results showed that LPS-induced intracellular ROS production was inhibited in FTL-overexpressing cells (Fig. 4B). This result is in accordance with a previous report\[25\].

3.5. FTL suppresses LPS-induced activation of MAPKs and NF-κB

Both MAPKs and NF-κB activation are essential for the expression of various pro-inflammatory genes\[26–28\]. To investigate whether FTL inhibits the LPS-induced inflammatory response by suppressing the activation of MAPKs and NF-κB, western blot analysis was performed on LPS-stimulated RAW264.7 murine macrophages. Fig. 5 shows that incubation of cells with LPS (100 ng/ml) for 30 min led to a 9-fold increase in p-p44/42 (p-ERK1/2) and a 6.5-fold in p38 phosphorylation. However, LPS-induced phosphorylation of p44/42 and p38 was dramatically inhibited in pFTL cells (Fig. 5A) and increased in shFTL cells (Fig. 5B). The phosphorylation level of IκBα (p-IκBα) significantly increased after LPS (100 ng/ml) stimulation for 30 min. Overexpression of FTL (pFTL) reduced the p-IκBα level. IκBα was dramatically degraded after LPS treatment for 30 min, which was partially recovered in pFTL cells (Fig. 5C). In contrast, knockdown of FTL by shFTL promoted LPS-induced IκBα phosphorylation and degradation (Fig. 5D). Additionally, the intracellular localization of the NF-κB p65 subunit was detected using an immunofluorescence assay. Overexpression of FTL could inhibit the nuclear translocation of NF-κB p65 in LPS-treated RAW264.7 cells (Fig. 5E).

4. Discussion

As a potent activator of the immune system, LPS induces a variety of immune responses. Particularly important is the massive activation of the cytokine cascades in macrophages, which results in severe infection response such as septic shock and SIRS that have high mortality. Here, we analyzed the role of FTL in the regulation of the mediators of inflammation. We used RAW264.7 cells and not macrophage primary cultures because RAW264.7 cells have a transfection efficiency that is 1000-fold better than macrophage primary cultures. TNF-α and IL-1β are two pro-inflammatory cytokines that play a pivotal role in the host immune response\[29\]. TNF-α is the most important initiator of the cytokine cascades. When cytokine production increases to such an extent that it moves outside of the local infection, sepsis or SIRS ensues. IL-1 is a potent inflammatory cytokine with various biological activities involved in regulating host defense and the immune response\[30\]. NO and PGE2, which play central roles in the pathogenesis of diverse inflammatory and infectious disorders\[31,32\].

![Fig. 4. FTL suppresses the LPS-induced increase of intracellular LIP and ROS. (A) Mean calcein fluorescence intensity was detected by a fluorescence plate reader at an excitation wavelength of 488 nm and emission of 517 nm in RAW264.7 cells after treatment with LPS (100 ng/ml) for 30 min. The amount of intracellular iron bound to calcein was assessed by addition of the cell-permeable iron chelator, SIH. Data are mean ± SEM (n = 5). **, p < 0.01, compared with untreated control cells; $$$, p < 0.001, compared with PBS-treated cells. (B) The exogenous Flag-FTL bound iron was measured by immunoprecipitation with anti-Flag antibody and inductively coupled plasma mass spectroscopy (ICP-MS, Thermo Fisher, X Series, FL, USA). $$$, p < 0.001, compared with PBS-treated cells. (C) TfR1 expression in shFTL cells was determined by western blot analysis. β-actin was used to verify equal loading in each lane. (D) The pcDNA3 or pFTL-transfected RAW264.7 cells were treated with PBS (control) or 100 ng/ml LPS (30 min), and the mean DCF fluorescence intensity was detected by flow cytometry at an excitation wavelength of 488 nm. **, p < 0.01, compared with untreated pcDNA3-transfected cells; $, p < 0.05, compared with LPS-treated pcDNA3-transfected cells. The experiments were repeated three times and similar results were obtained. The figure shows a representative result.](image-url)
Fig. 5. FTL suppresses LPS-induced activation of MAPKs and NF-κB. (A) The pFTL and pcDNA3 RAW264.7 clones were treated with or without LPS (100 ng/ml) for 30 min. The protein levels of p-p44/42, p44/42, p-p38 and p38 were determined by western blot analysis. ***, p < 0.001, compared with untreated pcDNA3 cells; **, p < 0.01, compared with LPS-treated pFTL cells. β-actin was used to verify equal loading in each lane. (B) The shFTL and pRNA-U6.1/neo (control) clones of RAW264.7 cells were treated with or without LPS (100 ng/ml) for 30 min. The p-p44/42, p44/42, p-p38 and p38 protein levels were tested using western blot analysis. ***, p < 0.001, compared with untreated pRNA-U6.1/neo cells; ***, p < 0.001, compared with LPS-treated shFTL cells. β-actin was used to verify equal loading in each lane. (C) The pFTL and pcDNA3 RAW264.7 clones were treated with or without LPS (100 ng/ml) for 30 min. Both p-IκBα and IκBα protein expression was determined by western blot analysis. ***, p < 0.001, **, p < 0.01, compared with untreated pcDNA3 cells; ***, p < 0.001, **, p < 0.01, compared with LPS-treated pFTL cells. β-actin was used to verify equal loading in each lane. (D) The shFTL and pRNA-U6.1/neo clones of RAW264.7 cells were treated with or without LPS (100 ng/ml) for 30 min. The protein levels of p-IκBα and IκBα were tested by western blotting. **, p < 0.01, *, p < 0.05, compared with untreated pRNA-U6.1/neo cells; **, p < 0.01, compared with LPS-treated shFTL cells. β-actin was used to verify equal loading in each lane. (E) Localization of NF-κB was analyzed by double immunofluorescence staining of NF-κB p65 subunit (green) and nucleus (blue). Immunofluorescence images were acquired by using an Axio Imager A2 microscope. All magnifications are 200×. The experiments were repeated three times and similar results were obtained. The figure shows a representative result.
are two important inflammatory mediators that play critical roles in LPS-induced inflammation and damage in addition to TNF-α and IL-1β. NO, which is synthesized from arginine by iNOS, is over-produced by activated macrophages and is important in the pathogenesis of septic shock and inflammatory tissue injury. PGE2 is a primary product of arachidonic acid metabolism, and over-production has been shown to play a crucial role in initiating systemic inflammatory response during sepsis [33]. PGE2 is synthesized via COX-2 [15]. Production of NO and PGE2 can cause pain, fever, swelling, and tenderness [34]. Thus, for treatment of severe infection, it is important to inhibit the LPS-induced production of TNF-α, IL-1β, NO and PGE2 to block the early cytokine cascades. 

In the present study, the FTL protein level was upregulated in RAW264.7 cells, a murine macrophage-like cell line, after 6 h stimulation with LPS. This result suggests that FTL may specifically mediate LPS-triggered inflammatory signaling in RAW264.7 cells. We first showed that overexpression of FTL inhibits LPS-induced transcription of TNF-α and IL-1β as well as expression of iNOS and COX-2. Furthermore, FTL inhibited the release of TNF-α, IL-1β, NO and PGE2 from RAW264.7 cells. All these data were confirmed by FTL RNA interference experiments. These data suggest that FTL acts as an anti-inflammatory factor in the LPS-induced inflammatory response.

Proper cellular iron handling and storage are the roles of ferritin, and any malfunction may cause iron mishandling that can lead to pathological consequences. Mutations in the FTL gene make the molecule susceptible to oxidation and cause hereditary ferritinopathy and neurodegeneration [35–37]. Free iron acts as a catalytic agent for ROS and results in iron-mediated oxidative stress [23,34]. Although LPS-induced oxidative stress is well-documented, the source and the mechanism of ROS formation remain elusive. Based on our findings, the mechanism that likely accounts for the anti-inflammatory action of FTL involves the inhibition of LPS-induced intracellular LIP upregulation, as well as ROS production, through iron sequestration. In the present study, overexpression of FTL reduced the free iron concentration and subsequent ROS production. Thus, FTL competed with the Fenton reactions and acted as an antioxidant because of the iron sequestration ability of exogenous FTL (Fig. 4B). Transient transfection of FTL shRNA resulted in a decreased expression of transferrin receptor protein 1 (TfR1) (Fig. 4C), which is a molecule essential for iron uptake. These data suggest that knockdown of FTL reduces ferritin complex and the free iron concentration is upregulated. The increase of intracellular free iron inhibited TfR1 expression.

Our data also showed the downregulation of FTL within the first 4 h of LPS stimulation. LPS stimulation directly increases NAPDH oxidase activity, which is associated with an increased intracellular ROS level [38]. Interestingly, FTL seems to be more sensitive to oxidation [39]. The FTL content is decreased due to the oxidation of intracellular ferritin, and the consequent proteasomal degradation reduced the ferritin content [17,39–42]. The experiment with the proteasomal inhibitor MG-132 (presented in supplementary Fig. 1) provided evidence that the proteasome is instrumental in the turnover of FTL. As a consequence of FTL degradation, an expansion of the free iron pool is likely to occur [42,43]. Iron plays a critical role during this process because it catalyzes the generation of free radicals formed from hydrogen peroxide via the Fenton reaction. Basal ROS production is boosted by expansive iron released from ferritin and accelerates ferritin degradation [39,43]. At the same time, the increased availability of iron induces ferritin gene transcription [42,43]. The specific iron chelator SIH inhibited LPS-induced protein expression of FTL (supplementary Fig. 2). Excess iron, caused by the increase of the ferritin synthetic rate, may allow reconstitution of ferritin content in an attempt to limit iron bioavailability. The high level of FTL following recovery might represent an adaptive response to oxidative stress because ferritin may be a protein with antioxidative properties [44,45]. It is possible that the synthesis of ferritin may not only return to normal levels but also increase at later time points. We hypothesized that the basal ROS production induced by LPS caused FTL degradation and cooperated to increase intracellular free iron levels within the first 4 h of LPS stimulation. This expansion of the iron pool induces the accumulation of more ferritin during the recovery period in RAW264.7 cells in the attempt to limit iron bioavailability.

Many previous reports indicated that ROS production was required for LPS-induced activation of MAPKs and NF-κB and the subsequent production of inflammatory cytokines [45–52]. Not surprisingly, we further found that FTL overexpression suppressed LPS-induced ERK, p38 and NF-κB activation.

In conclusion, our results reveal a novel role for FTL as an anti-inflammatory factor in the cellular response to LPS and provide new insight for understanding the detailed mechanisms of FTL regulation of the LPS-triggered inflammatory response. Thus, FTL may not only be a major iron storage protein but also an important regulator of the inflammatory response. Though previous reports showed that the induction of FTL synthesis was mediated by NO [53,54], TNF-α [55], IL-1β [56] and oxidative stress [25], the other factors that control the FTL expression level and inflammatory mediator production during activation of RAW264.7 cells remain to be investigated. Our study indicates a potent anti-inflammatory role for FTL in inflammatory diseases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.06.015.

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