Metalloreductase Steap3 coordinates the regulation of iron homeostasis and inflammatory responses

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Online Supplementary Design and Methods

Animals and treatment

Steap3$^{-/-}$ mice were obtained from Dr. Mark Fleming (Harvard Medical School and Children's Hospital, Boston, USA) and maintained on the C57BL/6J background; they were genotyped following previously reported protocols.[1] All animals were housed under specific pathogen-free conditions and provided with a standard rodent laboratory diet from SLRC Laboratory Animal Co. Ltd. (Shanghai, China) with an iron content of around 230 mg/Kg. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, and Chinese Academy of Sciences. Eight-week old C57BL/6J male wild-type mice or *Steap3*$^{-/-}$ mice were injected intraperitoneally with a single dose of lipopolysaccharide (LPS; 5 μg/g of body weight) or the same volume of phosphate-buffered saline as a control. Blood and tissues were harvested for either iron or gene expression assays at indicated time points. The control mice were sex- and age-matched and had a *Steap3*$^{+/+}$ genotype.

Primary cell isolation, culture, and treatment

Cultures of bone marrow-derived macrophage (BMDMs) were performed as described previously.[2] Matured BMDMs were washed and cultured in RPMI 1640 supplemented with 10% fetal bovine serum overnight, then treated with 100 mM ferric ammonium citrate (FAC) for 24 h, or were stimulated with 100 ng/mL LPS (from *Escherichia coli* 0111:B4, Sigma) or 5 μg/mL poly(I:C) (Sigma) for indicated times. In some experiments, 100 μM FAC were applied to the cell culture medium for 24 h before LPS stimulation. BMDMs were harvested for the calcein assay or in Trizol reagent (Invitrogen) for the measurement of gene mRNA levels or directly lysed for protein and iron assays.

For the collection and treatment of peritoneal macrophages, C57BL/6J mice were injected intraperitoneally with 4% fluid thioglycollate broth for 72 h,[3] then macrophages were collected from peritoneal cavities and cultured overnight before stimulation with LPS (100 ng/mL) for 6 or 12 h.

Mouse primary hepatocytes were isolated and cultured as described previously.[4] Isolated hepatocytes were used directly for the calcein assay or incubated in 10% fetal bovine serum-DMEM overnight and treated with or without FAC (100 mM) for western blotting or inductively coupled plasma mass spectrometry (ICP-MS). Some cells were cultured in medium from matured BMDMs before being stimulated with 100 ng/mL LPS for the indicated times, or cultured in macrophage-conditioned medium for the indicated times.

For macrophage-conditioned media experiments, BMDM were treated with LPS (100 ng/mL) or without LPS overnight and media were collected as macrophage-conditioned media. Twenty-five percent macrophage-conditioned medium plus 75% free medium were used to culture macrophages and hepatocytes for the indicated times. Cells were then collected for mRNA purification.

Measurements of serum iron and tissue non-heme iron

Serum iron concentrations were determined using a serum iron-unsaturated iron binding capacity kit (Thermo DMA) according to the manufacturer's instructions. Tissue non-heme iron was measured quantitatively as previously described.[5] Tissues were weighed and digested in acid solution (HCl/TCA) for 48 h at 65-70°C, and then A(535) was measured on 10 μL samples using a SpectraMax 190 (Molecular Devices, US) by the chromogen method. The results are presented as micrograms of iron per gram of wet tissue weight.

Total iron measurement with inductively coupled plasma mass spectrometry

BMDMs and hepatocyte lysates from *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice were collected and added to 200 μL HNO_3, then heated to 150 °C for at least 3 h. These samples were diluted with deionized water to a 3 mL final volume for total iron measurement with an Agilent 7500cx ICP/MS system (Agilent Technologies, Tokyo, Japan).[6] The results are presented as micrograms of iron per gram of total protein.

Cellular iron assay with scanning transmission X-ray microscopy

The spatial distribution of iron in cells was determined by scanning transmission X-ray microscopy analysis carried out at beamline 08U1-A in the Shanghai Synchrotron Radiation Facility. BMDMs from control and *Steap3*$^{-/-}$ mice were fixed in 70% ethanol overnight. The sample positioned on a high resolution X–Y–Z–θ stage was analyzed by the detector. The X-ray beam energy was scanned around the absorption K-edge of iron, between 708.5 and 702eV with 33 steps (0.2 eV step), and

then 708.5 and 707.5 eV were selected for iron standard analysis. Finally, the image was transferred to the computer for data acquisition and analysis.[7]

Calcein assay for intracellular iron concentrations

Mature BMDMs were harvested and hepatocytes were immediately separated and then stained with 0.5 μM calcein-AM (Molecular Probes) for 30 min before being subjected to flow cytometric analysis using a BD FACSCalibur.[8]

RNA extraction and quantitative reverse transcriptase polymerase chain reaction analysis

RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was performed as described previously.[2] Results were normalized to the internal control β-actin and presented as relative expression levels calculated by the $2^{\Delta\Delta}$Ct method and shown as mean±SEM. The qRT-PCR primers for *β-Actin*, *Steap1*, *Steap2*, *Steap3*, *Steap4*, *Hamp1*, *IFN-β*, *MCP-5*, *IP-10*, *RANTES* are listed in *Online Supplementary Table S1*.

Western blot analysis

Total protein (20-40 μg) was resolved on 10% sodium dodecylsulfate polyacrylamide gels. Blotting was performed with the following antibodies: rabbit anti-H-ferritin (1:1000 dilution, Alpha Diagnostics International), rabbit anti-L-ferritin (1:1000 dilution, Abcam), rabbit anti-pSTAT1 (1:1000 dilution, cell signaling), rabbit anti-β-actin (1:2000 dilution, Sigma), followed by either anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase at a dilution of 1:5000 (Proteintech Group, Inc.) and detected using the ECL System (Pierce). Western blots were quantified using Quantity One (Bio-rad) according to the manual.

Measurement of cytokines

Interleukin-6 and tumor necrosis factor-α levels in mouse sera and culture media were measured by enzyme-linked immunosorbent assays (R&D Systems) according to the manufacturer's instructions.

Ferrireductase activity assay

The reductase activity was measured as described previously.[9] Mature BMDMs and hepatocytes were washed with phosphate-buffered saline (pH=7.2) and incubated in iron uptake buffer with 50 μM Fe^{3+}-NTA. We used 200 μM ferrozine as an indicator of ferrous iron and monitored the increase in absorbance at 562 nm using an extinction coefficient of 27.9 $mM^{-1}cm^{-1}$ for the Fe^{2+}-ferrozine complex.

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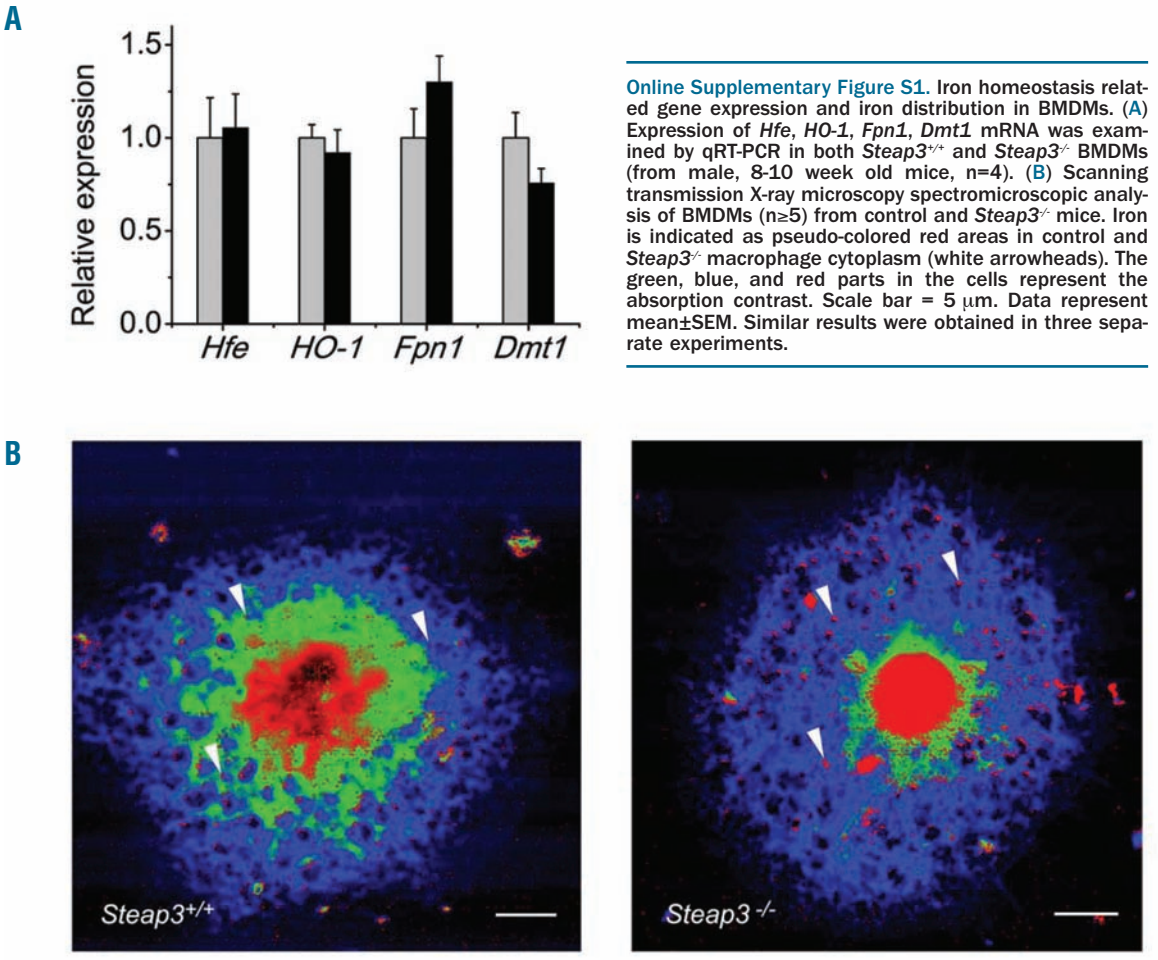
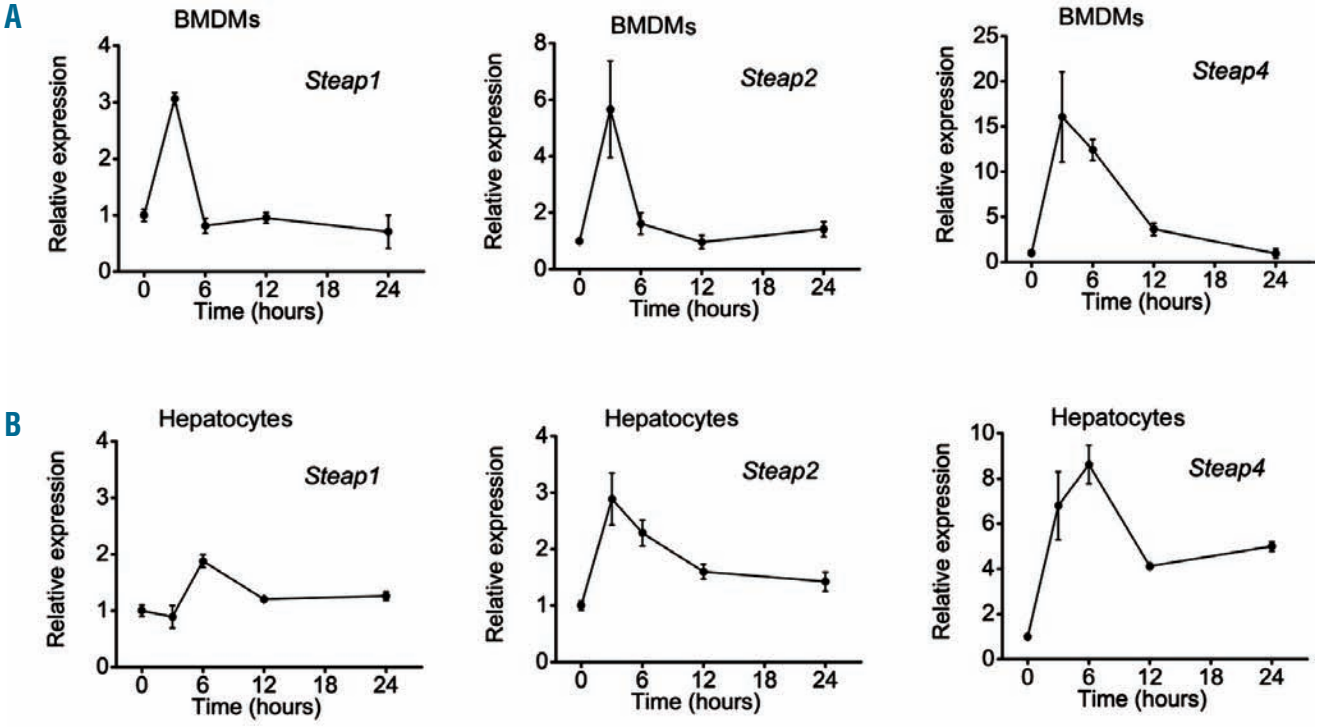
Online Supplementary Table S1. Sequences of oligonucleotide primers.

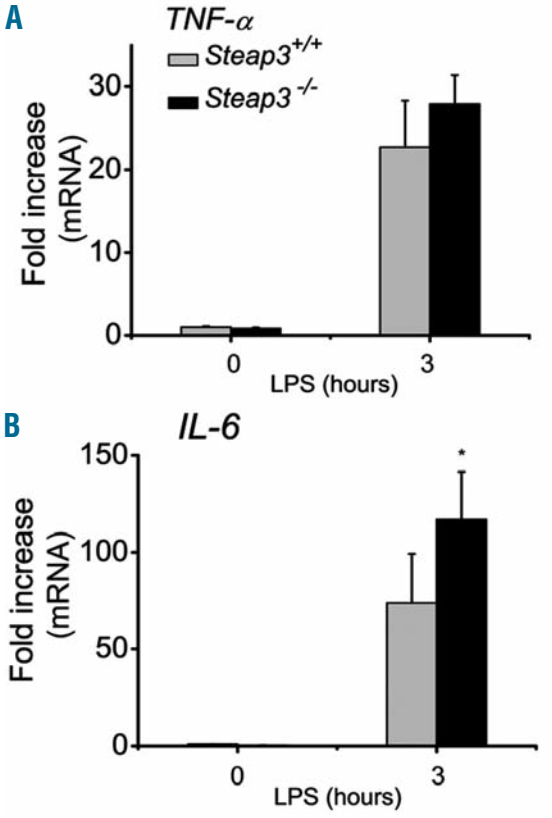
Gene name	Forward primer	Reverse primer	The primer reference
β-Actin	AAATCGTGCGTGACATCAAAGA	GCCATCTCCTGCTCGAAGTC	Current study
Steap1	GGTCGCCATTACCCTCTTGG	GGTATGAGAGACTGTAAACAGCG	Wellen *et al.*, 2007[10]
Steap2	TCGTTTTGCCCTCCATTGTA	CCCAGCCCTTTTTAATTCGTT	Wellen *et al.*, 2007[10]
Steap3	CCC GTC CAT TGC TAATTC CCT	CAG AAA AGA GAC CCGAAC CCA	Wellen *et al.*, 2007[10]
Steap4	TCA AAT GCG GAA TACCTT GCT	GCA TCT AGT GTT CCTGAC TGG A	Wellen *et al.*, 2007[10]
Steap3-exon2	TCTTCAGCACCGCCAGTCTAA	CTGTAGGGCCCATGCAGAGA	Current study
Hamp1	GCACCACCTATCTCCATCAACA	TTCTTCCCCGTGCAAAGG	Current study
IFN-β	GGCGGACTTCAAGATCCCTAT	TGGCAAAGGCAGTGTAACTCTTC	Current study
MCP-5	AGCTTTCATTTCGAAGTCTTTG	CTCCTTATCCAGTATGGTCC	Tuaillon *et al.*, 2002[11]
IP-10	GTGTTGACATCATTGCCACG	GCTTACAGTACAGAGCTAGG	Tuaillon *et al.*, 2002[11]
RANTES	GCGGGTACCATGAAGATCTCTG	CACTTCTTCTCTGGGTTGGCAC	Tuaillon *et al.*, 2002[11]



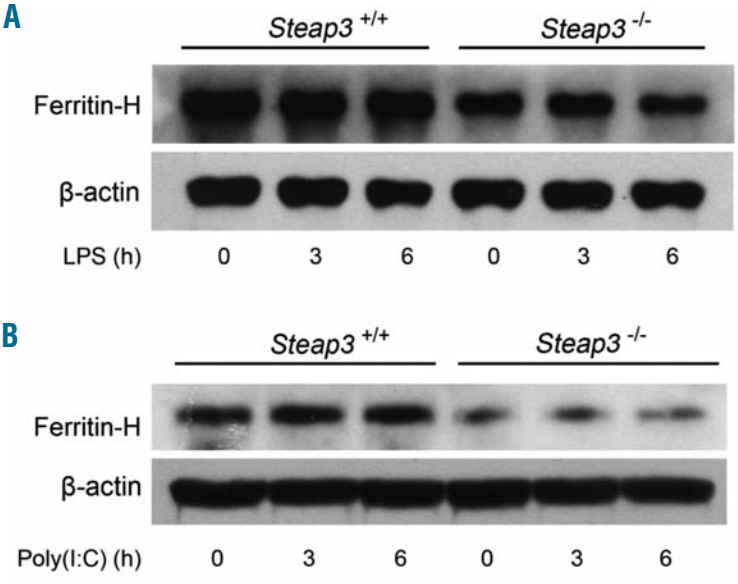
Online Supplementary Figure S1. Iron homeostasis related gene expression and iron distribution in BMDMs. (A) Expression of *Hfe*, *HO-1*, *Fpn1*, *Dmt1* mRNA was examined by qRT-PCR in both *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ BMDMs (from male, 8-10 week old mice, n=4). (B) Scanning transmission X-ray microscopy spectromicroscopic analysis of BMDMs (n≥5) from control and *Steap3*$^{-/-}$ mice. Iron is indicated as pseudo-colored red areas in control and *Steap3*$^{-/-}$ macrophage cytoplasm (white arrowheads). The green, blue, and red parts in the cells represent the absorption contrast. Scale bar = 5 μm. Data represent mean±SEM. Similar results were obtained in three separate experiments.



Online Supplementary Figure S2. *Steap1*, *Steap2* and *Steap4* expression in BMDMs and hepatocytes after LPS stimulation. (A) The mRNA levels of *Steap1*, *Steap2* and *Steap4* in BMDMs stimulated by LPS for the indicated times were measured by qRT-PCR. (B) mRNA levels of *Steap1*, *Steap2* and *Steap4* in hepatocytes stimulated by LPS for the indicated times were measured by qRT-PCR. Data represent mean ± SEM. Similar results were obtained in three separate experiments.



Online Supplementary Figure S3. *TNF-α* and *IL-6* mRNA levels in liver after LPS stimulation. *TNF-α* (A) and *IL-6* (B) mRNA levels in both *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice livers after LPS stimulation for 3 h were measured by qRT-PCR. Data represent mean ± SEM. Similar results were obtained in three separate experiments.



Online Supplementary Figure S4. *Steap3*$^{-/-}$ macrophages have low cytosolic available iron. BMDMs from control and *Steap3*$^{-/-}$ mice were stimulated with (A) 100 ng/mL LPS and (B) 5 μg/mL poly(I:C) for 3 or 6 h, and then cells were harvested and ferritin-H protein levels measured by western blot. Similar results were obtained in three separate experiments.