Lysosomal iron recycling in mouse macrophages is dependent upon both LcytB and Steap3 reductases

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Iron that is stored in macrophages as ferritin can be made bioavailable by degrading ferritin in the lysosome and releasing iron back into the cytosol. Iron stored in ferritin is found as Fe$^{3+}$ and must be reduced to Fe$^{2+}$ before it can be exported from the lysosome. Here we report that the lysosomal reductase Cyb561a3 (LcytB) and the endosomal reductase six-transmembrane epithelial antigen of prostate 3 (Steap3) act as lysosomal ferrireductases in the mouse macrophage cell line RAW264.7 converting Fe$^{3+}$ to Fe$^{2+}$ for iron recycling. We determined that when lysosomes were loaded with horse cationic ferritin, reductions or loss of LcytB or Steap3 using CRISPR/Cas9-mediated knockout technology resulted in decreased lysosomal iron export. Loss of both reductases was additive in decreasing lysosomal iron export. Decreased reductase activity resulted in increased transcripts for iron acquisition proteins DMT1 and transferrin receptor 1 (Tfrc1) suggesting that cells were iron limited. We show that transcript expression of LcytB and Steap3 is decreased in macrophages exposed to Escherichia coli pathogen UTI89, which supports a role for these reductases in regulating iron availability for pathogens. We further show that loss of LcytB and Steap3 in macrophages infected with UTI89 led to increased proliferation of intracellular UTI89 suggesting that the endolysosomal system is retaining Fe$^{3+}$ that can be used for proliferation of intravesicular pathogens. Together, our findings reveal an important role for both LcytB and Steap3 in macrophage iron recycling and suggest that limiting iron recycling by decreasing expression of endolysosomal reductases is an innate immune response to protect against pathogen proliferation and sepsis.

Key Points

- Lysosomal reductase activity is necessary for iron release and recycling.
- Loss of lysosomal reductases increases the proliferation of intracellular pathogens.

Introduction

Iron plays an essential role in enzymatic reactions that drive heme, iron-sulfur cluster, lipid, and DNA synthesis. The majority of total body iron (3-4 g) can be found as hemoglobin in red blood cells (2-3 g). Red blood cells have a life span of ~100 to 120 days necessitating a mechanism for iron recycling from senescent red cells. Macrophages play a central role in iron recycling from senescent or damaged red cells by delivering about 20 to 30 mg of iron back into the bloodstream daily for further rounds of utilization. Macrophages in the liver and spleen engulf most of the aged red cells, and the resulting phagosome fuses with the lysosome where the red cell is degraded. As the red blood cell is degraded, heme released into the lysosomal lumen is then transported out of the lysosome by HRG1. Cytosolic heme is catalyzed by heme oxygenase-1 releasing Fe$^{2+}$ into the cytosol that can then be exported out of macrophages through ferroportin (Fpn) or stored in cytosolic ferritin. Under iron-limited conditions, ferritin in the...
macrophages can be engulfed in an autophagosome through recruitment of the cargo receptor NCOA4\textsuperscript{10,12} which then fuses with the lysosome.\textsuperscript{13} In the lysosome, ferritin is broken down, and Fe\textsuperscript{3+} is released into the lysosomal lumen where it can be stored or transported back into the cytosol through divergent metal transport 1 (DMT1), Natural resistance–associated macrophage protein 1 (NRAMP1) or transient receptor potential mucolipin 1 (TRPML1).\textsuperscript{14,15} Because iron transporters identified to date accept Fe\textsuperscript{3+} only as a substrate, there must be a mechanism to convert lysosomal Fe\textsuperscript{3+} to Fe\textsuperscript{2+} before it can be exported to the cytosol. To date, a mammalian lysosomal ferrireductase has not been described for macrophage iron recycling. One candidate that is predicted to be a macrophage lysosomal ferrireductase is Cyb561a3 (referred to here as LcytB), which has been localized to lysosomes in the RAW264.7 mouse macrophage cell line, although its function as a reductase for iron was not demonstrated.\textsuperscript{16} LcytB is a member of the cytochrome b561 (Cyt b561) family, which is a group of intrinsic membrane proteins involved in ascorbate-mediated transmembrane electron transport.\textsuperscript{17} An example of a Cyt b561 reductase is Dcytb, which acts in iron absorption in the gut converting Fe\textsuperscript{3+} to Fe\textsuperscript{2+} for iron import at the apical surface of the gut epithelium.\textsuperscript{18} Saccharomyces cerevisiae also expresses plasma membrane ferrireductases Fre1 and Fre2, and the absence of both results in poor growth on iron-limited media.\textsuperscript{19}

The poor growth of \(\Delta fre1\Delta fre2\) yeast can be partially complemented by overexpression of mammalian LcytB, but this complementation requires plasma membrane localization of LcytB,\textsuperscript{20} whereas LcytB is localized to lysosomes in macrophages.\textsuperscript{16} A recent article showed that transcripts for the gene encoding LcytB (Cyb561a3) were dramatically increased when lysosomal acidification was blocked and that this increase was a result of cellular iron limitation.\textsuperscript{21} This suggests that LcytB is important in iron release from lysosomes, although this has not been directly shown.

The six-transmembrane epithelial antigen of prostate 3 (Steap3) is an endosome-localized ferrireductase that works with DMT1 to export iron out of the endosome.\textsuperscript{22} Previous studies have shown that Steap3 partner transporter DMT1 translocates to the lysosome upon ferritin loading,\textsuperscript{14} which suggests that Steap3 may also translocate to the lysosome of macrophages depending upon iron status and reductase need. Here we report that both LcytB and Steap3 are important in lysosomal iron release from macrophages. We generated deletions in LcytB, Steap3, and in both in RAW264.7 mouse macrophages. We show that preloading the lysosome with iron-laden horse spleen cationic ferritin (CF) (which is broken down in the lysosome) and iron is then released to the cytosol, which results in increased endogenous mouse cytosolic ferritin levels; the absence of LcytB or Steap3 decreases the release of lysosomal iron. We also show that the absence of both reductases is additive in iron release from lysosomes. We determined that Steap3 shows increased colocalization with the lysosomal marker Lamp2 when cells are fed CF. Finally, we show that loss of these reductases in RAW264.7 cells increases the proliferation of Escherichia coli pathogen UTI89, which can live in the lysosome, confirming that more iron is bioavailable for the survival and proliferation of vascular pathogens.

**Materials and methods**

**Cell culture**

The murine macrophage cell line RAW264.7 was cultured in Dulbecco’s modified Eagle medium (DMEM; Mediatech) containing 0.2 mM L-alanyl-L-glutamine dipeptide (Gibco), 0.1 mM sodium pyruvate (Sigma), 10% fetal bovine serum, and penicillin-streptomycin (Sigma) at 37 °C under 5% CO\textsubscript{2}. For ascorbate experiments, cells were incubated with 0.3 M ascorbate for 48 hours after CF loading. C57BL/6 bone marrow–derived macrophages were isolated and grown as previously described.\textsuperscript{23}

**Bone marrow–derived macrophages and RNA interference**

C57BL/6 bone marrow–derived macrophages were isolated and grown as previously described.\textsuperscript{23} Small interfering RNA (siRNA) pools matching selected regions of LcytB complementary DNA (cDNA) sequences and a random sequence pool were purchased from Thermo Fisher Scientific. Transfections were performed on bone marrow–derived macrophages plated at 50% confluence using electroporation (Amaxa Neon) with siRNA pools at a final concentration of 100 nm according to manufacturer’s directions. Cells were transfected twice (time 0 and time 24 hours) and then grown for an additional 48 to 72 hours before harvesting for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) or ferri- tin measurements.

**CRISPR/Cas9 generation and complementation of mutations**

LcytB and/or Steap3 mutants were generated by using a lentiviral CRISPR/Cas9 system. CRISPR constructs were generated under the guidance of Crystal Davey, PhD, Core Director, Mutation Generation Detection Core. The LcytB-CRISPR constructs were targeted to the sequence on exon 3 or exon 4 of the mouse cyb561a3, whereas the Steap3-CRISPR constructs were targeted to a sequence on exon 1 of the mouse Steap3. Reductase mutants were identified by high-resolution melting analysis, and changes in Cyb561a3 or Steap3 messenger RNA (mRNA) were confirmed by qRT-PCR. For LcytB complementation, the pCMV3-GFP Spark-human cyb561a3 plasmid (Sino Biological) was transfected into LcytB mutants using the Neon electroporation transfection system (Thermo Fisher Scientific) according to the manufacturer’s protocol for macrophages. The transfected cells with green fluorescent protein (GFP) were selected for analysis by flow cytometry (BD FACSaria) and kept in hygromycin 200 μg/mL for 2 weeks. For Steap3 complementation, the pCDNA6-Steap3-Myc-His construct (a generous gift from Mark Fleming, MD, Harvard University)\textsuperscript{22} was transfected into Steap3 mutants using the same transfection system as described above. The transfected cells were selected in 10 to 20 μg/mL blasticidin. All selected LcytB or Steap3 overexpression cell lines were then placed in antibiotic-free media to confirm stable expression before complementation analysis and to ensure that any observed effects were not the result of the presence of antibiotics.

**qRT-PCR**

Total RNA was harvested using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 2 μg RNA were used to make cDNA. Power SYBR Green Master Mix (Applied Biosystems) was used on a Mastercycler Realplex2 system (Eppendorf). Actin was used as a control housekeeping gene. The ΔΔCt method was used to compare the variation of transcripts among samples. Specificity and efficiency were checked before using this method. Primers for mouse genes included Actin forward...
Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The hemogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA, and 10 mM Trizma base [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The hemogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The hemogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]). Cells were homogenized using a ball-bearing homogenizer with a 0.2561-inch diameter ball. The homogenates were centrifuged at 400g at 4°C for 5 minutes, and the supernatant was centrifuged at 14,000g for 30 minutes. The pellet was solubilized in lysis buffer (1% Triton, 150 mM NaCl, 0.5 mM tetrasodium EDTA [pH 7.4]).
Figure 1.
analyses were performed on all graphs using either Grubbs or ROUT tests \((Q = 1.0)\) as recommended by Prism software. Identified outliers were removed from the plots and before the statistical analyses.

**Results**

**Loss of LcytB resulted in reduced lysosomal iron export**

Iron released from ferritin that is degraded in the lysosome is \(\text{Fe}^{3+}\) must be converted to \(\text{Fe}^{2+}\) to be exported, because all identified mammalian iron transporters transport divalent metals. This conversion requires the presence of a reductase on the lysosome. Previous studies have suggested that LcytB might be that reductase. To determine whether LcytB is the macrophage lysosomal reductase responsible for the conversion of \(\text{Fe}^{3+}\) to \(\text{Fe}^{2+}\) and subsequent lysosomal iron export, we used the mouse macrophage cell line RAW264.7 and CRISPR/Cas9 targeted mutagenesis of Cypb561a3, which encodes LcytB. We targeted either exon 3 or exon 4 of Cypb561a3, hereinafter referred to as LcytB. We identified 2 clones, LcytB \#1 and LcytB \#2, that have significantly reduced levels of LcytB transcripts (Figure 1A). Unfortunately, commercially available LcytB antibodies did not work for assessing LcytB protein levels in mouse samples. Both LcytB \#1 and LcytB \#2 showed a slower growth rate compared with WT cells (supplemental Figure 1A). These results suggest that LcytB may be important in regulating bioavailability of iron for proliferation. One measure of cellular iron status is cytosolic ferritin levels. To determine whether loss of LcytB affected cellular iron levels, we measured endogenous ferritin levels. When the cells were maintained under normal growth conditions, clones LcytB \#1 and LcytB \#2 had significantly lower endogenous ferritin compared with WT cells (Figure 1B, –CF). These results suggested that LcytB contributes to maintaining endogenous ferritin levels in macrophages. To determine whether the loss of LcytB affected iron release and recycling from lysosomes, we took advantage of a previously established method to load lysosomes with CF and measure iron release from lysosomes. This method uses horse spleen CF, which is taken up by fluid-phase endocytosis and delivered to the lysosome where it is degraded, and ferritin is either stored in the lysosome or transported into the cytosol (Figure 1C). The released iron is then either exported out of the cell through ferroportin or incorporated into newly synthesized cytosolic ferritin for storage. Importantly, the horse spleen CF is not recognized by antibodies against mouse ferritin that are part of the ELISA assays used to measure cellular mouse ferritin. Using CF allows us to distinguish between steady-state endogenous cytosolic ferritin and increases in ferritin synthesis upon lysosomal iron release. Indeed, loading with CF increased endogenous cytosolic ferritin levels in WT cells approximately twofold, whereas LcytB mutants showed a 50% to 70% reduction in cytosolic ferritin compared with CF-fed WT cells (Figure 1B, +CF). We confirmed that reductions in endogenous ferritin in LcytB-mutant cells were not a consequence of decreased fluid-phase endocytosis, which is required for CF uptake. Cells were incubated with fluorescently labeled Alexa594-dextran (10,000 molecular weight), and total fluorescence was determined using flow cytometry at the indicated time points. No differences were seen in the rate or amount of dextran taken up in WT vs LcytB-mutant cells (supplemental Figure 1B). This supports the notion that reductions in LcytB affect iron export out of lysosomes but not fluid-phase endocytosis.

To confirm that the changes in endogenous ferritin were a result of the loss of LcytB and not because of some other compensatory mutation in our CRISPR/Cas9 clones, we transfected LcytB-mutant cells with a plasmid containing a GFP-tagged human LcytB whose expression was driven by a cytomegalovirus (CMV) promoter. Previous studies have shown that overexpressed LcytB localizes with Lamp2 and Lysotracker-positive compartments reflecting late endosomes and lysosomes. We confirmed expression using immunofluorescence microscopy and showed that LcytB-GFP colocalizes with Lamp2, but not with endosomal reductase Steap3 (Figure 1D). We note that not all LcytB-GFP localizes with Lamp2. We speculate that this is because of overexpression and that LcytB-GFP may still be in the synthesis/secretory pathway en route to lysosomal localization. LcytB-GFP+ cells were sorted by flow cytometry, maintained in hygromycin to establish stable cell lines, and expanded in the absence of hygromycin for further use in ferritin assays. LcytB-mutant cells overexpressing LcytB-GFP showed increased levels of endogenous ferritin both with and without CF loading (Figure 1B, pLcytB (plasmid expressing WT human LcytB-GFP)), providing strong evidence that the reduction in endogenous ferritin levels seen in our mutant cell lines was the result of loss of LcytB and was not

![Figure 1. CRISPR/Cas9-mediated mutation of LcytB in RAW264.7 macrophages results in decreased iron release from lysosomes.](image)
Figure 2. Loss of Steap3 in macrophages results in decreased release of iron from lysosomes. (A) RNA was isolated from WT RAW264.7 cells, cDNA was generated, and qRT-PCR was performed for Steap2, Steap3, Steap4, and Actin. Data were normalized to a Steap3 level of 1.0 (ratio Steap3:Actin; n = 3 biological replicates). (B) RNA was isolated from WT, Steap3 #2, and Steap3 #13 cells, cDNA was generated, and qRT-PCR was performed for Steap3 and Actin. Data were normalized to a WT level of 1.0 (n = 3 biological replicates). (C) Membranes were isolated from biological replicates of WT, Steap3 #2, and Steap3 #13 cells, and western blot analysis was performed for Steap3 and Lamp2. A representative blot with 2 biological replicates is shown. Stds, standard molecular mass markers. (D) Western blots were quantified using Fiji Image J with Lamp2 as a loading control. Data were normalized to a WT level of 1.0 (n = 4 biological replicates). (E) WT, mutants Steap3 #2 and Steap3 #13, and Steap3 mutants
a result of off-target CRISPR/Cas9-generated mutations. We also determined that, although WT LcytB improves endogenous ferritin levels in the mutant LcytB #2 cell line, the Y66A-mutant LcytB reductase does not (Figure 1E).

Previous studies using overexpression in yeast have shown that LcytB is a reductase, and reductase activity was increased when yeast were provided exogenous ascorbate.20 Earlier studies also showed that addition of ascorbate to fibroblasts increased

Figure 3. Loss of LcytB and Steap3 is additive in limiting the export of lysosomal iron. (A) Steap3 was mutated in LcytB #2 as described in Figure 2. qRT-PCR analysis was performed on WT and DKD #1 and DKD #2 clones using Steap3 and Actin primers. Data were normalized to a WT level of 1.0. (n > 3-7 biological replicates). (B) Crude membrane preparations were performed for WT, DKD #1, DKD #2, and Steap3 #2, and western blot analysis for Steap3 and VDAC was performed. VDAC was used as a membrane loading control. A representative blot with 2 biological replicates is shown. (C) Western blots were quantified using Fiji Image J using VDAC as a membrane loading control. Data were normalized to a WT level of 1.0 (n = 4 biological replicates). (D) Cytosolic ferritin levels were determined in cells incubated in the presence or absence of CF as in Figure 2B. Data were normalized to a WT cytosolic ferritin level (–CF) of 1.0 (n ≥ 6 biological replicates). (E) WT cells were treated with or without CF as in Figure 1B and fixed. Steap3 and Lamp2 localization were detected by confocal immunofluorescence microscopy. A representative field is shown for –CF +CF. Arrows denote areas of Steap3 and Lamp2 colocalization in CF-loaded cells. (F) Data from 5 biological replicate slides were quantified, and the data were displayed as percentage of cells showing Steap3 and Lamp2 colocalization. More information on procedures is provided in “Materials and methods.” Error bars represent SEM. *P < .05; **P < .01; ***P < .001; ****P < .0001.
lysosomal iron export, which suggests that ascorbate was limiting for reductase activity. Ascorbate dependence has not been shown in macrophages. Surprisingly, the addition of ascorbate to WT RAW264.7 macrophages did not significantly affect ferritin levels in cells fed CF (Figure 1F). This result suggests that LcytB in macrophages is not an ascorbate-dependent reductase or that in the context of the lysosome, there is another electron donor that is available for reduction of Fe3+. We anticipated that loss of LcytB would prevent any ascorbate-dependent increases in cytosolic ferritin in CF-fed cells; however, our LcytB mutants showed increased cytosolic ferritin levels upon CF lysosome loading. This suggests that either there is some functional LcytB present on the lysosome or that there is another reductase that contributes to iron recycling from the lysosome. Our qRT-PCR results show that LcytB expression is dramatically reduced in our mutant cells (Figure 1A), which makes this possibility less likely. We focused on the possibility of the presence of another lysosomal reductase. Steap3 is a reductase that is present in the endocytic pathway and is expressed by macrophages. Steap3−/− mice show abnormal iron homeostasis with increased anemia. However, the latter phenotype was attributed to endosomal iron export in red cell precursors. We confirmed that Steap3 is expressed in the RAW264.7 macrophage cell line, whereas the homologous reductases Steap2 and Steap4 showed low levels of expression (Figure 2A). To determine whether Steap3 is important in iron recycling from the macrophage lysosome, we used CRISPR/Cas9 targeted mutagenesis to create Steap3-mutant RAW264.7 cells. We successfully mutated Steap3 and identified 2 clones, Steap3 #2 and

Loss of Steap3 resulted in reduced export of lysosomal iron

Loss of LcytB decreased the ability to export iron from the lysosome as measured by reductions in endogenous ferritin synthesis. The decrease that resulted from LcytB loss, however, was not complete, because cells still showed increased cytosolic ferritin upon CF lysosome loading. This suggests that either there is some functional LcytB present on the lysosome or that there is another reductase that contributes to iron recycling from the lysosome. Our qRT-PCR results show that LcytB expression is dramatically reduced (>90%) in our mutant cells (Figure 1A), which makes this possibility less likely. We focused on the possibility of the presence of another lysosomal reductase. Steap3 is a reductase that is present in the endocytic pathway and is expressed by macrophages. Steap3−/− mice show abnormal iron homeostasis with increased anemia. However, the latter phenotype was attributed to endosomal iron export in red cell precursors. We confirmed that Steap3 is expressed in the RAW264.7 macrophage cell line, whereas the homologous reductases Steap2 and Steap4 showed low levels of expression (Figure 2A). To determine whether Steap3 is important in iron recycling from the macrophage lysosome, we used CRISPR/Cas9 targeted mutagenesis to create Steap3-mutant RAW264.7 cells. We successfully mutated Steap3 and identified 2 clones, Steap3 #2 and

Figure 4. Loss of LcytB or Steap3 alters total levels of cellular metals. Metal levels were determined in WT, LcytB #2, Steap3 #2, and DKD #2 cells using ICP-MS. Graphs represent 3 to 5 biological replicates for whole cells. Data were normalized to protein levels (pg/μg protein). Fe (A), Cu (B), Mn (C), and Zn (D). *P < .05; **P < .01.
Steap3 #13, with significantly reduced Steap3 transcript levels (Figure 2B). Western blot analysis confirmed the presence of Steap3 protein in WT RAW264.7 cells and dramatically reduced Steap3 levels in selected Steap3 mutants (Steap3 #2 and Steap3 #13; Figure 2C-D). Steap3 mutants did not show any growth defect and may have a slight growth advantage compared with WT cells (supplemental Figure 2). Steap3 mutants also did not show any defect in endocytosis (supplemental Figure 1B), but they had less endogenous cytosolic ferritin compared with control RAW264.7 cells grown under normal cell culturing conditions (Figure 2E, -CF). Steap3 has previously been localized to endosomes and functions with DMT1 to reduce transferrin-delivered Fe\(^{3+}\) to Fe\(^{2+}\) for export from the endosome to the lysosome to export ferritin-released iron from the lysosome in hepatocytes when iron is limited by chelation treatment.\(^{30}\) To determine whether Steap3 also functions as a lysosomal reductase, we incubated Steap3 mutants and WT RAW264.7 cells with CF and measured endogenous ferritin level changes. When WT cells were incubated with CF, endogenous ferritin level increased as expected; however, the levels were significantly decreased in the Steap3 mutants (Figure 2E, +CF). Complementation analysis confirmed that the change in iron release from lysosomes was the result of reduced levels of Steap3, because overexpressing mouse Steap3-His expressed under a CMV promoter\(^{22}\) in Steap3 mutant cells increased ferritin levels closer to those of WT cells. We confirmed Steap3-His expression (Figure 2F, WT+, Steap3 #2+, Steap3 #13+) and noted that Steap3-His expression was higher in the Steap3 mutants (Steap3 #2 and Steap3 #13) compared with WT cells harboring the Steap3-His plasmid (WT+). This limited our ability to assess whether overexpression of Steap3 improves the export of lysosomal iron. We speculate that there may be more selective pressure to keep Steap3 in the mutant cell lines to control cellular iron levels. Our complementation results confirmed that the changes in ferritin levels in Steap3 mutants were a result of the loss of Steap3 and not an off-target effect of the CRISPR/Cas9-driven mutagenesis.

**Loss of LcytB and Steap3 is additive in reducing the export of lysosomal iron**

Loss of either LcytB or Steap3 reduced lysosome iron recycling by about 50%. We wondered whether these effects were additive. To answer this question, we mutated Steap3 in LcytB #2 using the same CRISPR/Cas9 strategy that was used to generate single
Steap3 mutations and selected 2 double knockdown (DKD) clones, DKD #1 and DKD #2, as determined by qRT-PCR (Figure 3A). We confirmed that the DKD clones showed significant reductions in Steap3 protein (Figure 3B-C). Loss of both LcytB and Steap3 resulted in a significant growth defect compared with WT cells (supplemental Figure 3), suggesting that the cells are limited for iron. We examined whether the loss of both LcytB and Steap3 affected iron release from CF breakdown in lysosomes compared with loss of LcytB or Steap3 alone. DKD clones contained significantly less cytosolic ferritin compared with LcytB or Steap3 mutants under normal cell culture conditions or when fed with CF (Figure 3D). Together, our results support that both LcytB and Steap3 function as reductases that are important in the release of iron from lysosomes. Because Steap3 has predominantly been ascribed to act in release of iron from endosomes, we wondered whether Steap3 could translocate to the lysosome. To test this hypothesis, we examined Steap3 localization in cells fed CF. If Steap3 is working in the endosome, we would expect to see no change in Steap3 localization upon CF lysosome loading. Conversely, if Steap3 localized to the lysosome upon lysosome iron loading, we would expect to see increased colocalization with the lysosomal marker Lamp2. Loading macrophages with CF resulted in increased colocalization of Steap3 with Lamp2 (Figure 3E); however, not all cells showed increased Steap3 colocalized upon CF loading (Figure 3F). Our data show that, similar to results reported for DMT1, some Steap3 localizes to the lysosome, thus permitting iron reduction and transport into the cytosol.

Loss of LcytB or Steap3 alters total levels of cellular metals
To determine whether the loss of LcytB or Steap3 affects iron levels, we used inductively coupled plasma mass spectrometry (ICP-MS) with whole-cell lysates. We determined that loss of Steap3, but not LcytB, reduced levels of whole-cell iron (Fe) (Figure 4A). We suggest that whole-cell Fe levels are reduced in the Steap3-mutant cells because RAW264.7 cells are highly dependent upon Tf(Fe)2/Tfrc1/Steap3–mediated cellular iron uptake. We speculate that whole-cell iron levels are not altered by loss of LcytB because the iron becomes sequestered within the lysosome. Loss of Steap3

Figure 6. Loss of LcytB and Steap3 alters iron acquisition transcripts. (A) WT cells were incubated with or without CF or grown in the presence or absence of DFO for 48 hours. qRT-PCR was performed for LcytB, Steap3, DMT1, Tfrc1, and Actin. Data are the ratio of Actin levels and are normalized to WT cells grown without CF or DFO as 1.0 (n = 4 or more biological replicates). (B) WT, LcytB-mutant, Steap3-mutant, and DKD cells were grown as in panel A and transcript levels for DMT1, Tfrc1, Fpn1, and Actin were measured using qRT-PCR. Data are the ratio of Actin levels and are normalized to WT cells under similar growth conditions (1.0) (n = 4 or more biological replicates). More information on procedures is provided in “Materials and methods.” Error bars represent SEM. *P < .05; **P < .01; ***P < .001; ****P < .0001.
also reduced total cellular copper (Cu) levels (Figure 4B), whereas loss of LcytB reduced manganese (Mn) levels (Figure 4C). No changes in the amounts of zinc (Zn) were observed (Figure 4D).

We do not know the reasons for these specific Cu and Mn changes in Steap3 and LcytB mutants. Interestingly, the loss of both reductases resulted in recovery of WT whole-cell levels of Fe, Cu, and Mn. We speculate that this may be a result of upregulation of plasma membrane metal transporters in response to loss of both reductases as a means to meet cellular demands for these metals.

**Loss of lysosomal reductases affects the expression of other ferrireductases**

Macrophages express several reductases that can reduce multivalent metals. We examined whether the loss of LcytB or Steap3 affected the expression of other ferrireductases. We anticipated that some reductases may be upregulated in response to loss of LcytB or Steap3. Surprisingly, loss of LcytB decreased expression of Steap3 (Figure 5A). Similarly, loss of Steap3 decreased LcytB transcript levels. We determined that RAW264.7 cells express low levels of Steap2 and Steap4 (Figure 2A) compared with LcytB and Steap3 levels, and these transcripts were unchanged by loss of either LcytB or Steap3 (Figure 5B). However, both Steap2 and Steap4 expression were increased upon loss of both LcytB and Steap3. These results suggest that the expression of the endolysosomal localized reductases LcytB and Steap3 are tightly coordinated. When those reductases are absent, macrophages increase the expression of other reductases to increase bioavailable iron, either through acquisition of external iron or release of iron from the endolysosomal system.

**Loss of LcytB or Steap3 alters iron acquisition gene expression**

We hypothesized that LcytB levels might be affected by cellular iron levels, which we modified by either loading cells with...
lysosomal iron or by growing cells under iron limitation using the iron chelator desferoxamine (DFO). qRT-PCR analysis showed that LcytB transcripts were increased when the lysosome was loaded with iron (CF loading) and decreased when cells were limited for iron by growth in DFO (Figure 6A). Steap3 transcripts were unaffected by CF or DFO. Similar to Steap3, DMT1 transcripts were unaffected. To ensure that the iron limitation and CF-loading growth conditions were changing cellular iron status, we measured transferrin receptor 1 (Tfrc1) transcripts. It is known that under iron-limited conditions, iron regulatory proteins 1 and 2 (IRP1 and IRP2) bind to the 3’ untranslated regions of Tfrc1 mRNA, which contain iron regulatory elements to stabilize the mRNA. As expected, CF-loading resulted in decreased Tfrc1 transcripts, whereas iron limitation (DFO) dramatically increased Tfrc1 transcripts. That DMT1 expression was unchanged upon altered cellular iron levels in RAW264.7 macrophages confirms observations previously reported for the mouse macrophage cell line J774. We wondered whether expression of these iron acquisition genes, DMT1 and Tfrc1, was affected in our LcytB- and Steap3-mutant RAW264.7 cells. In response to loss of the reductase LcytB, DMT1 transcripts levels were increased; however, no changes in DMT1 transcripts were seen when Steap3 was absent (Figure 6B). The absence of both LcytB and Steap3 significantly increased DMT1 expression beyond that seen in the absence of LcytB alone. Furthermore, Tfrc1 transcripts were also upregulated in the absence of reductases LcytB and Steap3, but the loss of both was not additive for Tfrc1. Transcripts for the exporter ferroportin Fpn1 were significantly decreased in the individual LcytB #2 and Steap3 #2 mutants but, intriguingly, this effect was negated in the absence of both reductases. We do not have an explanation for this observation at this time. Our results indicate that loss of LcytB or Steap3 results in limited bioavailable iron, which is sensed by the RAW264.7 macrophages and results in the upregulation of iron acquisition genes.

Transcript levels of Steap3 and LcytB are altered upon exposure to pathogens

Macrophages are the major iron recycling cells in mammals, and when iron is abundant, macrophages also store iron in the cytosolic protein ferritin. In iron-limited conditions, macrophages recycle iron from stored intracellular ferritin. Macrophages are a key cell type in innate immunity, providing nutrients to tissues near sites of infection and limiting nutrient availability to opportunistic pathogens. Previous studies determined that Steap3 transcripts were decreased when bone marrow–derived macrophages were exposed to the lipopolysaccharide present on many pathogens. This finding suggests that macrophages respond to the presence of extracellular pathogens by signaling through MyD88 to limit iron release from the lysosome. To test whether LcytB expression is also altered by exposure to pathogens, we used the pathogenic E. coli strain UTI89. We incubated WT RAW264.7 cells with or without UTI89 and examined LcytB and Steap3 transcript levels. Both LcytB and Steap3 transcripts were decreased by >50% upon exposure to UTI89 (Figure 7A). These results suggest that macrophages have an innate immune response to bacterial exposure to limit intracellular iron release by reducing expression of reductases involved in iron release from lysosomes.

Loss of LcytB or Steap3 improves proliferation of intracellular pathogen UTI89

Exposure to pathogens can cause cellular iron retention and sequestration in macrophages as a way to limit iron availability for extracellular pathogens. Under these conditions, intracellular (or facultative intracellular) pathogens such as E. coli, Salmonella typhimurium, Mycobacterium tuberculosis, Chlamydia psittaci, and Legionella pneumophila are known to thrive. Organisms that live intracellularly in macrophages have evolved mechanisms to evade immune recognition and still have access to essential host intracellular iron. We questioned whether reductions in LcytB and/or Steap3 affected the ability of pathogens to establish an intracellular niche. We infected WT RAW264.7, LcytB #2, Steap3 #2, and DKO #2 cells with the pathogenic E. coli strain UTI89, which can reside in a vesicular compartment in macrophages, and we assessed invasion and proliferation. We did not observe any differences in bacterial adhesion that resulted from the loss of LcytB or Steap3 (Figure 7B). At T0, 30 minutes after inoculation, loss of LcytB or Steap3 led to increased intracellular titers of UTI89, although this was statistically significant only for the LcytB mutant (Figure 7C). Loss of both LcytB and Steap3 did not have an additive effect. In overnight assays (T0/N), the LcytB #2 and Steap3 #2 both showed significantly increased numbers of bacteria compared with WT cells. In these longer assays, loss of both LcytB and Steap3 had an additive effect, resulting in markedly higher intracellular bacterial titers than those observed in the WT macrophages (Figure 7D). Together, these results indicate important roles for the LcytB and Steap3 reductases in limiting bacterial survival within macrophages. It is important to note that E. coli strains such as UTI89 can take up Fe³⁺ by siderophore secretion and by expressing bacterial reductases. Thus, Fe³⁺ retained in the endolysosomal system may be used by bacteria for proliferation. Our results support that limiting endosome and lysosome ferrireductase activity may provide a growth advantage to pathogens that survive in the endocytic pathway by retaining iron within endolysosomal membranes.

Discussion

Macrophages are key to iron recycling from red cells and iron release from stored ferritin. When iron demand is high, macrophages engulf ferritin through autophagy, ferritin is degraded in the lysosome, and iron is released as Fe³⁺ into the lysosomal lumen. All known mammalian iron transporters are Fe²⁺ transporters, including those present on the lysosome. Thus, iron export requires a lysosomal reductase to convert Fe³⁺ to Fe²⁺ that can then be exported to the cytosol for Fe²⁺ export back to plasma. Here, we provide evidence that the lysosomal protein LcytB, a predicted ferrireductase, is important in iron release from lysosomal-degraded ferritin in macrophages. Loss of LcytB, using CRISPR/Cas9 mutagenesis, reduced lysosomal iron export by about 50% in RAW264.7 macrophages. That there was still some iron export from the lysosome suggested that there was another reductase that could act in the absence of LcytB. We confirmed that reduction in Steap3 decreased ferritin-released lysosomal iron export by approximately 50%, and the effects were additive in reducing iron export from the lysosome. Previous studies have reported that LcytB is the
predominant lysosomal reductase in Burkitt B cells, but that other B-cell lymphomas rely on Steap3. This observation, together with results reported here, suggests that LcytB dependence may vary between cell types. We observed that Steap3 contributed to iron release from lysosomal localized ferritin and demonstrated that, similar to reports for DMT1, some Steap3 localized to the lysosome. In CF-loaded cells, only a small portion of Steap3 was found colocalized with Lamp2. One explanation for the small amount of colocalization is that Steap3 may be working at both the endosome and lysosome when lysosomal iron levels were increased. This possibility could occur through fusion of endosomes and lysosomes during cargo delivery (for review, see Luzio et al) where “kiss and run” between lysosomes and endosomes would provide rapid additional reductase activity to reduce iron and permit transport out of the endocytic pathway under high iron demand. In either case, we provide evidence that both LcytB and Steap3 contribute to lysosomal iron export.

Our LcytB/Steap3 DKD cells still showed a small increase in the level of cytosolic ferritin after CF loading. Remaining iron export from the lysosome can be explained either by remaining LcytB or Steap3 activity or by the existence of another reductase that can function in the absence of LcytB and Steap3. It is also possible that there are reducing equivalents present in the lysosome that can convert Fe$^{3+}$ to Fe$^{2+}$, as suggested by La et al. We observed that Steap2 and Steap4 expression levels were increased in response to the loss of both LcytB and Steap3. Both Steap2 and Steap4 have been localized to the endocytic pathway, and we predict that they can contribute to the endosomal lysosomal iron export under limited LcytB and Steap3 reductase activities. To determine whether Steap2 and Steap4 contribute to lysosomal iron recycling will require the generation of quadruple-mutant cells lines, which is beyond the scope of this study.

LcytB is predicted to be an ascorbate-dependent reductase in which ascorbate provides the electron to reduce Fe$^{3+}$ to Fe$^{2+}$ in our studies, incubation of RAW264.7 mouse macrophages with ascorbate did not alter intracellular ferritin levels upon CF loading, which is different from that observed in fibroblasts. In addition, we were surprised to find that our LcytB mutants grown in the presence of ascorbate showed an increase in intracellular ferritin levels upon CF loading. Our results suggest that ascorbate is altering cellular homeostasis independent of LcytB-mediated lysosomal iron reduction. The addition of ascorbate to growth medium is complicated to interpret because (1) ascorbate may reduce media Fe$^{3+}$ to Fe$^{2+}$ that can then be transported through plasma membrane DMT1 into the cytosol resulting in increased cellular ferritin levels, (2) ascorbic acid has been reported to inhibit lysosomal autophagy of ferritin, (3) ascorbate has been speculated to stabilize ferritin or increase ferritin synthesis, and (4) increasing ascorbate levels have been suggested to provide electron donors in the cytosol that can reduce ferritin iron from Fe$^{3+}$ to Fe$^{2+}$, allowing iron release directly into the cytosol. In all of these cases, one might expect to see changes in macrophage ferritin levels independent of LcytB status. Future studies are required to tease out the consequences of altering cellular ascorbate levels and how ascorbate affects iron homeostasis in macrophages.

One prediction of loss of either LcytB or Steap3 is that whole-cell iron levels will change. Interestingly, we observed that loss of LcytB did not affect iron levels, whereas loss of Steap3 reduced amounts of whole-cell iron by about 50%. We suggest that there were no changes in whole iron in LcytB-mutant cells because the iron becomes sequestered within the lysosome. The changes observed in Steap3-mutant cells may mean that RAW264.7 macrophages are highly dependent upon Steap3 for iron acquisition via Tfrc1-Tf(Fe)$^{2+}$ uptake and Steap3-mediated endosomal reduction of Fe$^{3+}$ to Fe$^{2+}$ before export out of the endosome through DMT1. It was surprising to find that Mn and Cu levels were reduced upon loss of LcytB and Steap3, respectively. It has been shown that Steap3 can act as a Cu reductase and that Fe and Cu metabolism are tightly regulated. Thus, Steap3 loss may reflect a decrease in Cu uptake through the Cu transporters Ctr1 and Ctr2. We speculate that the change in Mn levels in LcytB-mutant cells may reflect competition for metal transport through DMT1 or other metal transporters (such as NRAMP1 or TRPML1), which are known to transport several different divalent metals. Why Mn is specifically reduced in the absence of LcytB remains to be determined. Perhaps our most intriguing finding was that loss of both LcytB and Steap3 restores normal levels of these metals within the whole cells. We observed that DMT1 transcripts were upregulated about twofold by loss of both LcytB and Steap3 compared with loss of LcytB alone, whereas Tfrc1 expression was not significantly altered in the absence of both LcytB and Steap3 in comparison with the single reductase deletions. These observations suggest that elevated DMT1 levels may allow for increased cellular Fe, Mn, and Cu uptake needed for cellular functions and survival. Further analysis of the uptake of these metals and their subcellular localization may provide additional insight into how LcytB and Steap3 impact the use and storage of metals by macrophages.

It is known that macrophages retain iron to sequester it away from extracellular pathogens. Some pathogens have taken advantage of this to survive and acquire iron inside of macrophages. We found that U189, a uropathogenic E. coli strain, which can reside within a vesicular compartment in macrophages, persisted and proliferated better in our mutant lines LcytB #2, Steap3 #2, and DKD #2 RAW264.7 macrophages. These observations support the hypothesis that there is more endolysosomal iron available for pathogen proliferation in the absence of LcytB. We and others have observed that Steap3 and LcytB expression is reduced in response to pathogen exposure. This suggests that these reductases may be part of the innate immune response in macrophages that limits nutrient bioavailability during infection. We speculate that these reductases may also contribute to macrophage-mediated iron delivery to the surrounding tissue environment. Future studies are focused on determining the contribution of LcytB to both innate immune regulation and macrophage function.

In conclusion, our studies have identified the reductases LcytB and Steap3 as important players in the conversion of Fe$^{3+}$ to Fe$^{2+}$ within the lysosome, allowing for Fe$^{3+}$ export back to the macrophage cytosol. Steap3 mutations have been identified in humans and result in microcytic anemia, and the Steap3 knockout mouse shows retention of iron in macrophages and anemia, although the levels are reduced by only approximately 60%. These observations suggested that a reductase other than Steap3 is important for iron recycling and iron homeostasis. Here, we provide evidence that lysosomal LcytB acts as an additional macrophage reductase important in ferritin iron recycling. No known mutations in LcytB have been reported in humans, and future studies regarding the role of
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Authorship

Contribution: F.M. performed experiments, generated figures, and helped write the manuscript; B.A.F. designed and performed experiments and edited the manuscript; X.J. performed experiments and edited the manuscript; A.A.R. performed mutagenesis of pLcytB-GFP; M.A.M. designed experiments and edited the manuscript; and D.M.W. designed and performed experiments, generated figures, and helped write the manuscript.

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