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Macrophage-specific LAMTOR2 deletion disrupts systemic iron homeostasis in mice

Laura Homs Pérez^{1,2}, Iana Portnaia^{1,2}, Markus Seifert^{1,2}, Sylvia Berger¹, Stephanie Waich³, Lukas Müller¹, Nidhi Srinivasan¹, Lukas A. Huber³, Martin Offterdinger⁴, Richard Hilbe¹, Alexander Hoffmann^{1,2}, Manuel Grander^{1,2}, David Haschka^{1*}, Günter Weiss^{1,2*}.

¹Department of Internal Medicine II, (Infectious Diseases, Immunology, Rheumatology, Pneumology)

²Christian Doppler Laboratory for Iron Metabolism and Anemia Research, Medical University of Innsbruck, Innsbruck, Austria

³Institute of Cell Biology, Biocentre, Medical University of Innsbruck, Innsbruck, Austria

⁴Institute for Systemic neuroscience - Bio-optics core facility, Medical University of Innsbruck, Innsbruck, Austria

Correspondence

Günter Weiss and David Haschka, Department of Internal Medicine II, Medical University of Innsbruck, Anichstr. 35, A-6020 Innsbruck, Austria, email: Guenter.weiss@i-med.ac.at or david.haschka@i-med.ac.at

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ABSTRACT

At the center of iron homeostasis, macrophages play a crucial role by phagocytosing senescent erythrocytes while nursing erythrocyte progenitors. A major route of cellular iron acquisition occurs via transferrin receptor 1 (TFR1)-mediated endocytosis of transferrin-bound iron, a process that critically depends on endosomal trafficking. The late endosomal adaptor LAMTOR2 regulates mTORC1/MAPK signaling and endosomal traffic, yet its role in macrophage iron handling is unknown.

Here we show that mice lacking LAMTOR2 in myeloid cells develop iron-restricted erythropoiesis, characterized by reduced hepcidin and bone marrow iron deficiency along with increased erythrophagocytosis and splenic iron accumulation, and compensatory extramedullary erythropoiesis. Macrophages deficient in LAMTOR2 display markedly reduced TFR1 protein and impaired transferrin-mediated iron uptake. Mechanistically, loss of LAMTOR2 results in shifting of TFR1 to lysosomes which were hyperacidified when compared to control cells. Pharmacological inhibition of lysosomal acidification but not of proteasomal degradation restores TFR1 in LAMTOR2-deficient macrophages.

Our study collectively identifies a novel role for the LAMTOR2 complex in preventing TFR1 degradation and potentially facilitating iron delivery from bone marrow macrophages to erythroid progenitors. We demonstrate that LAMTOR2-dependent endosomal trafficking is a critical, previously unrecognized mechanism that stabilizes the transferrin receptor, enables essential iron recycling in macrophages and ultimately sustains systemic iron homeostasis.

INTRODUCTION

Daily, we require around 20 to 30 mg of iron to sustain healthy erythropoiesis and metabolism. However, only 1 to 2 mg are absorbed in the duodenum; most iron originates from splenic red pulp macrophages (RPMs) and Kupffer cells that phagocytose senescent red blood cells (RBCs) and export recycled iron to the circulation via the iron exporter ferroportin (FPN1) to peripheral cells or the bone marrow (BM).¹⁻⁷ In the BM, a central macrophage is surrounded by multiple erythroblast progenitors forming an erythroblastic island. The nursing macrophage assists in the development of the erythroblasts by directly interacting and providing growth factors, supplying iron and engulfing their nuclei in the last stage of development.^{1, 8, 9}

At systemic level, the hormone hepcidin is the master regulator of iron homeostasis. Upon iron overload, hepcidin is produced in the liver and downregulates FPN1 on the basolateral membrane of the enterocytes or macrophages, thereby blocking duodenal iron absorption and resulting in macrophage iron retention.^{3, 10, 11} In contrast, iron deficiency, hypoxia or anemia reduce liver hepcidin expression, thereby enforcing dietary iron absorption, iron re-distribution from macrophages and thus enhancing circulating iron levels.^{3, 10-12}

When ferric iron (Fe^{3+}) enters the bloodstream, it binds to transferrin (Tf). Circulating Tf-bound iron (holo-Tf) interacts with transferrin receptor 1 (TFR1) and the complex is internalized via clathrin-mediated endocytosis.^{13, 14} The affinities of both Tf-TFR1 complex and iron-Tf binding are pH dependent.^{15, 16} On the cell membrane at physiological pH, holo-Tf exhibits high affinity for TFR1. Upon reaching acidic endosomal compartments, Fe^{3+} is released from Tf and the resulting apo-Tf-TFR1 is re-exposed to the cell surface. There, the binding affinity decreases and apo-Tf is dissociated from TFR1 to the circulation.^{13, 17, 18} Dissociated Fe^{3+} in the endosomes is reduced to ferrous iron (Fe^{2+}) by six-transmembrane epithelial antigen of the prostate 3 (STEAP3) and transported into the cytoplasm through divalent metal transporter 1 (DMT1).¹⁹⁻²¹

TFR1 is either recycled via recycling endosomes, or directly from early endosomes to the cell membrane.^{22, 23} This canonical recycling pathway ensures a rapid and continuous supply of iron to the cells. However, another route has been identified, in which TFR1 fuses with lysosomes, leading to degradation of the receptor complex. This pathway post-translationally reduces TFR1 in response to elevated intracellular iron.^{24, 25}

Like all the other cell types, macrophages require iron to support cellular metabolism. Iron is incorporated in heme-containing enzymes, cytochromes of the electron transport chain, and iron-sulfur cluster-containing proteins associated with mitochondrial respiration, DNA synthesis, and other functions.¹ Iron acquisition pathways vary among macrophage subtypes. Although all macrophage populations express TFR1, RPMs and Kupffer cells acquire mainly heme iron through erythrophagocytosis and the relative contribution of TFR1 mediated iron uptake in specific macrophages subtypes has not been comparatively studied thus far.²⁶

LAMTOR2 belongs to the LAMTOR/Ragulator complex [late endosomal and lysosomal adaptor and MAPK and mTOR (mammalian target of rapamycin) activator], which is

composed of 5 subunits (LAMTOR1 to 5).²⁷ This complex serves primarily as a platform for two very important signaling pathways: MAPK signaling²⁸ and mammalian target of rapamycin complex 1 (mTORC1) functionality.²⁹ LAMTOR2 specifically, has been shown to participate in the biogenesis, architecture and function of the endo/lysosomal system, EGFR degradation,^{28, 30} and in the efficient transport of phagocytosed bacteria through the endosomal system.³¹ Moreover, LAMTOR2 has been implicated in Langerhans cell homeostasis,³² it is required for early embryogenic development,³³ and it has been involved in focal adhesion dynamics during cell migration,³⁴ among other functions.

Given the well-established role of macrophages in iron recycling and erythropoiesis, and the recognized functions of LAMTOR2 within the endo-lysosomal system — including its significance in cellular iron acquisition via TFR1 — we examined whether LAMTOR2, when expressed specifically in macrophages, is vital for maintaining systemic iron homeostasis and erythropoiesis in mice.

METHODS

Animals

Animal experiments were approved by the Medical University of Innsbruck and the Austrian Federal Ministry of Science and Research (BMFWF-2023-0.203.946). We used 8 to 12-week-old male LysMCre LAMTOR2^{+/+} (LMC) and LysMCre LAMTOR2^{-/-} (LAMTOR2 KO) mice on a C57BL/6 background.³¹ The generation of the targeting construct and the conditional allele of LAMTOR2 were achieved by Teis, Taub³³. Mice received a standard iron diet (180 mg of iron/kg, Ssniff, Soest, Germany). The mice were sacrificed, and blood, liver, spleen, duodenum, and BM extracted. Additional details can be found in the *Online Supplementary Appendix*.

Blood count and plasma measurements

Blood count was performed using scil VET abc (Scil animal care company GmbH, Viernheim, Germany) blood counter. For the remaining blood, plasma was extracted by centrifugation at 200 G for 20 min at room temperature (RT) and taking the supernatant. From the plasma, iron was quantified using the QuantChrome™ Iron Assay (BioAssay Systems, Hayward, CA, USA) following the manufacturer's protocol. Hepcidin and erythropoietin (EPO) were measured with ELISAs (HMC-001 Hepcidin Murine-Compete ELISA Kit, Intrinsic LifeSciences, La Jolla, CA, USA; Mouse Erythropoietin Quantikine ELISA Kit, R&D, Minneapolis, MN, USA).

Iron quantification methods in cells and tissues

Tissue iron content was measured using acid-hydrolysed tissue homogenates through a colorimetric assay involving bathophenanthroline disulfonate and L-ascorbic acid in a sodium acetate-based reaction, as described elsewhere.⁶ The obtained iron was normalized to the protein concentrations of each sample, which were determined using the Bradford assay.

For Perl's Prussian Blue Staining, histological analyses of spleen and liver tissues on formalin-fixed sections were stained. Images were acquired using an Olympus

BX61VS slide scanner (Olympus, Tokyo, Japan) equipped with a 20x objective (numerical aperture 0.75) and processed with OlyVIA software (Olympus). Further details as well as information on atomic absorption spectrometry (AAS), determination of the cytosolic labile iron pool (LIP) by calcein quenching, determination of free Fe²⁺ iron by the FerroOrange methods in any cellular compartment and by LysoFerroRed, to measure lysosomal free Fe²⁺ iron can be found in the *Online Supplementary Appendix*.

Production and seeding of bone marrow-derived macrophages (BMDMs)

Production, differentiation and seeding of BMDMs was performed as previously described³⁵ and additional details can be found in the *Online Supplementary Appendix*.

Transferrin uptake studies

BMDMs were incubated with Alexa Fluor™ 488–conjugated Tf for 20 min at 37°C (Thermo Fisher). For flow cytometry, cells were stained for CD45 (BD Biosciences), F4/80 (BioLegend), and CD11b (BioLegend), and Tf uptake was quantified as FITC median fluorescence intensity (MFI) in live CD45⁺F4/80⁺CD11b⁺ macrophages. For immunofluorescence, cells were fixed, permeabilized, stained with phalloidin and DAPI, and imaged by fluorescence microscopy. For more details, see the *Online Supplementary Appendix*.

Lysosomal pH determination

For lysosomal and pH staining *in vivo*, we used the Lysosomal Acidic pH Detection Kit (Dojindo, L266) and followed the protocol provided. For more details, see the *Online Supplementary Appendix*.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software). Data are presented as mean ± standard deviation (SD). Normality was assessed by Shapiro-Wilk test. Depending on the distribution/variance, groups were compared using Student's t-test, Welch's t-test, or Mann-Whitney U test; two-way ANOVA with Tukey's post hoc test was used for multifactor comparisons. Outliers were identified using the ROUT (Q = 1%).

RESULTS

LAMTOR2 deletion in the myeloid line alters systemic iron homeostasis

LMC and LAMTOR2 KO mice³¹ were characterized to investigate the role of LAMTOR2 in iron metabolism and homeostasis. First, we performed a complete blood analysis to assess red blood cell counts (RBCs) (*Figure S1A*), hemoglobin (*Figure S1B*), hematocrit (*Figure S1C*), mean corpuscular volume (MCV) (*Figure S1D*), mean corpuscular hemoglobin concentration (MCHC) (*Figure S1E*), mean corpuscular hemoglobin (MCH) (*Figure S1F*), and red cell distribution width (RDW) (*Figure S1G*). Reticulocyte counts were determined by flow cytometry (*Figure 1A*, *Figure S1H*). The general blood count analysis did not reveal any differences between the two groups.

In contrast, we found a significant increase in reticulocytes percentage in LAMTOR2 KO mice, indicating increased erythropoiesis.

Of note, monocyte numbers and percentage in the blood, (Figure S2A, B) and macrophage numbers in the BM (Figure S2C) were also assessed, revealing that their numbers were not different between the two genotypes.

We next assessed iron-regulatory and erythropoietic parameters. Serum hepcidin was reduced in LAMTOR2 KO mice (Figure 1C) in parallel to decreased hepatic *Hamp* mRNA (Figure 1D). In contrast, serum EPO (Figure 1E), ERFE (below detection, data not shown) and iron (Figure 1F) did not differ significantly, although serum iron showed an increased trend ($p = 0.0521$) in LAMTOR2 KO mice, consistent with reduced hepcidin and increased transfer of iron from cells to the circulation.

Notably, the spleens of LAMTOR2 KO mice were enlarged, as quantified by increased spleen weight (Figure 1B).

Subsequently, iron in the liver, spleen, duodenum, and BM was assessed by colorimetric quantification and Perl's Prussian Blue staining. Our results showed that total iron was significantly higher in LAMTOR2 KO spleens (Figure 2B), whereas no difference was observed in iron among the other organs investigated (Figure 2A, C and D). Perl's Prussian blue staining revealed higher non-heme iron in LAMTOR2 KO liver (Figure 2E; Figure S2F) and spleen (Figure 2E; Figure S2D) while less iron was detected in the BM (Figure 2E; Figure S2E).

These data suggested that LAMTOR2 KO mice suffer from a defective iron distribution, leading to BM iron depletion and liver/splenic iron retention.

Further, we performed Western blots of whole-organ lysates from liver, spleen and BM samples to assess protein levels of TFR1, FPN1 and ferritin (FT). Our results showed that TFR1 and FPN1 were higher in LAMTOR2 KO liver and spleen (Figure 3A; Figure S3A, B), but lower in the BM (Figure 3A; Figure S3C) when compared to wild-type mice. Additionally, FT was higher in LAMTOR2 KO spleens (Figure 3A; Figure S3B), which is consistent with their elevated iron content. Contrary to expectation, FT was also increased in the BM (Figure 3A; Figure S3C). These protein changes indicate systemic iron dysregulation in LAMTOR2 KO mice.

To investigate the impact of previous findings on erythropoiesis in LAMTOR2 KO mice, we used flow cytometry to quantify the following erythroid progenitor populations: basophilic, polychromatic and orthochromatic.³⁶ As shown in Figure 3B (and Figure S5A), BM basophilic erythroblast percentage increased in LAMTOR2 KO mice while orthochromatic progenitors were decreased, indicative of reduced erythroid maturation. Characterization of the BM by RT-PCR revealed that erythropoietin receptor (*Epor*), Janus kinase 2 (*Jak2*), signal transducer and activator of transcription 5 (*Stat5*), and BCL2-like 1 (*Bcl2l1*) (Figure S4A-D) (involved in erythroid survival and maturation) were downregulated, in parallel with *Tfr1* (Figure S4E), and *Dmt1* (Figure S4F) (involved in iron uptake). We then analyzed the erythroid progenitor populations in the spleen by flow cytometry, as well (Figure S5B). Surprisingly, all three populations were extensively increased in LAMTOR2 KO mice, indicating that extramedullary erythropoiesis is stimulated, most likely to compensate for the reduced BM erythropoiesis, consistent with the increased spleen size (Figure 1I).

In summary, these data suggest that LAMTOR2 deficiency in myeloid cells, restricted to monocytes, macrophages, and neutrophils, disrupts systemic iron homeostasis in mice. This is characterized by a pathological iron retention in the spleen, decreased iron stores in the BM, and defective erythropoiesis in the BM. In response, the mice activate extramedullary erythropoiesis in the spleen.

LAMTOR2 KO downregulates TFR1 and leads to decreased Tf uptake

To study the mechanism leading to dysfunctional iron handling by macrophages, we generated BMDMs from LMC and LAMTOR2 KO mice. For that, we incubated them under control conditions and upon supplementation with 25 μ M of Ferric nitrate nonahydrate (FeN) for 8 and 24 h and analyzed the protein expression of TFR1, FPN1 and FT. Most strikingly, TFR1 was markedly reduced in LAMTOR2 KO BMDMs under all conditions (Figure 4A; *Figure S6A*). In addition, there was a tendency towards higher FT and FPN1 expression in LAMTOR2 KO specifically upon iron supplementation and incubation over 24 h (Figure 4A; *Figure S6, C*)

Given the marked reduction of TFR1 found in LAMTOR2 KO cells, we next investigated the consequences for Tf uptake. For that, we used two different approaches: fluorescent microscopy (Figure 4B, C) and flow cytometry (Figure 4D, E; *Figure S7A, B*). For both experiments, Tf-AF488 was incubated for 20 min. Consistent with the diminished TFR1 expression, levels of Tf fluorescence were significantly lower in LAMTOR2 KO BMDMs in both setups.

Furthermore, intracellular iron was measured using different approaches: atomic absorption spectrometry (AAS) (Figure 5A), to quantify the total amount of iron in cells; Calcein-AM (Figure 5B), to determine the cytosolic labile iron pool (LIP); FerroOrange, to quantify free Fe^{2+} iron in any cellular compartment (Figure 5C; *Figure S8A*); and LysoFerroRed, to measure lysosomal free Fe^{2+} iron (Figure 5D, E). As shown, no differences were found in intracellular iron using AAS nor Calcein-AM, while FerroOrange showed increased free Fe^{2+} iron in the KO BMDMs. Notably, both LAMTOR2 KO BMDMs and RPMs (*Figure S8B, C*) showed significantly increased levels of lysosomal iron, suggesting that lysosomal iron mobilization appears to be impaired in LAMTOR2 deficient macrophages.

Along with these experiments, we performed a Tf-AF488 pulse-chase in BMDMs to examine whether Tf recycling was impaired in LAMTOR2 KO cells as well. To assess this, we tracked the uptake of Tf-AF488 over a 20-min period, harvesting the cells at 0, 5 and 15 min. The harvested cells were labelled for the endosomal markers early endosome antigen 1 (EEA1) and lysosome-associated membrane protein 1 (LAMP1). As in previous studies, Tf uptake was severely impaired in LAMTOR2 KO cells (*Figure S9A, B*). At the 0-min time point, almost no Tf was detected on the cell surface. This deficiency persisted at 5 and 15 min, with significantly less intracellular Tf observed in KO cells than in LMC. Consequently, the low levels of internalized Tf in LAMTOR2 KO cells precluded a definitive analysis of intracellular recycling, as meaningful co-localization with EEA1 or LAMP1 could not be assessed. Notably, the distribution of LAMP1 in these cells was also altered, shifting from a perinuclear localization to a more peripheral pattern, as previously observed.³³

Altogether, these results indicate that TFR1 downregulation in LAMTOR2 KO BMDMs reduces Tf uptake without altering the total iron or LIP, whereas lysosomal iron content increases.

TFR1 is missorted to the lysosomes where it degrades

Next, we investigated the cause of TFR1 downregulation in LAMTOR2 KO BMDMs. Since TFR1 is known to be degraded in both the proteasome and the lysosome as a post-translational mechanism,^{24, 25, 37} we exposed cells to inhibitors specific to each pathway: Bafilomycin (BAF), which inhibits lysosome acidification, and bortezomib

(BORT), which inhibits proteasomal degradation. We also incubated cells with HUWE E3 inhibitor (BI8622), which is known to ubiquitinate TFR1 to be shifted to the proteasome³⁷ (*Figure S10A*).

TFR1 expression in LAMTOR2 KO cells remained unaffected by the presence of bortezomib or a HUWE1 E3 inhibitor (*Figure 6C, D; Figure S10A*). By contrast, BAF increased TFR1 protein expression in both LMC and KO BMDMs (*Figures 6A, B*), with no corresponding change in *Tfr1* mRNA (*Figure S10B*).

To further explore the link between the reduced TFR1 protein expression and lysosomal function, we examined whether the lysosomal acidification was altered in LAMTOR2 KO BMDMs. Previous studies have shown that deletion of individual LAMTOR components lead to the degradation of the entire LAMTOR complex and that the other components were rapidly degraded via the proteasome.^{27, 38} Since the LAMTOR complex regulates lysosomal signaling and pH homeostasis, we hypothesized that its loss could impair lysosomal acidification, thereby influencing TFR1 stability. For that, lysosomes were stained in green with LysoPrime and pH was visualized with pH LysRed. As a negative control, BAF, was also used. As shown in *Figure 7A and B*, normalization of both dyes revealed that LAMTOR2 KO BMDMs exhibited a markedly higher pH LysRed/LysoPrime Green, indicating enhanced lysosomal acidification compared to control cells. These results indicate that LAMTOR2 KO lysosomes are hyperacidified.

Finally, we investigated whether TFR1 was missorted in the lysosomes in LAMTOR2 KO BMDMs thereby contributing to its degradation. For that, we stained TFR1 and LAMP1 and incubated the cells with iron, BAF, or both (BAF and FeN). As shown in *Figure 8A*, lysosomes in LAMTOR2 KO BMDMs appeared expanded throughout the cytoplasm, whereas LMC lysosomes were located perinuclear. TFR1 decreased after addition of iron in both genotypes, as expected, and increased after addition of BAF in both genotypes as well. When both, BAF and iron, were added, levels of TFR1 remained high in both LAMTOR2 and LMC. Interestingly, co-localization in steady-state conditions remained constant between LAMTOR2 KO and LMC. Upon addition of BAF and BAF plus FeN, co-localization in LAMTOR2 KO cells was significantly higher (*Figure 8B*), suggesting that TFR1 is missorted to the lysosomes when LAMTOR2 is missing.

Taken together, these results suggest that LAMTOR2 deletion of BMDMs results in reduced TFR1 expression which can be traced back to TFR1 shuttling to lysosomes and accelerated breakdown due to reduced pH.

Discussion

To our knowledge, this is the first study that identified a direct link between the LAMTOR complex, iron metabolism, and erythropoiesis.

Systemically, we found a wide range of iron-associated physiological changes in the KO mice. Iron accumulated in the liver and the spleen, where TFR1, FPN1 and FT (only in the spleen) expression was increased. Contrary, iron depletion was detected in the BM, together with decreased TFR1 and FPN1. We also observed reticulocytosis, decreased erythroid progenitor maturation in the BM, along with expanded extramedullary erythropoiesis in the spleen, reduced hepcidin serum levels, and higher serum iron.

This is a complex phenotype that differs from the canonical functional iron-deficiency phenotype characterized by elevated hepcidin, reduced FPN1, hypoferremia, and blunted iron export from spleen and liver.³⁹ Mechanistically, decreased hepcidin in the LAMTOR2 KO mice may arise from an increased demand for RBC production, suggested by the reticulocytosis and dysfunctional BM erythropoiesis observed, leading to erythropoiesis mediated downregulation of hepcidin. However, our findings showed that serum EPO was not elevated in the KO mice. Thus, we assumed that another pathway could be involved. We then studied ERK1/2, STAT3, and SMAD 1/5/8 in the liver and, as shown in *Figure S11*, pERK1/2 was downregulated in the KO mice. Although there was considerable variability within replicates, the overall quantification revealed a significant decrease.

Next, high FPN1 levels in the liver and spleen may be the result of low circulating hepcidin levels and/or activation of NRF2 signaling. The latter notion arises from our finding of increased expression of the Nrf2 target genes BCL2-like 1 (*Bcl2l1*), *Fpn1*, FT heavy chain 1 (*Fth1*), and heme oxygenase 1 (*Hmox1*) in KO mice, whereas expression of FT light chain 1 (*Ftl1*) and glutathione peroxidase 4 (*Gpx4*) (*Figure S12*) were not altered. These observations indicate that NRF2 is induced, possibly in response to increased iron accumulation in these organs with subsequent induction of oxidative stress.

In regard to the elevated iron depositions in the spleen despite having high FPN1 levels, we propose that: 1) iron is trapped in splenic macrophages within FT (which is also high), or in their hyperacidic lysosomes. In *Figure S13A-B*, we show that Prussian Blue-stained iron fits with the localization of F4/80⁺ macrophages. In parallel, we found that lysosomal free ferrous iron in KO BMDMs and isolated RPMs was significantly increased; 2) that iron import is higher than export.

Further, we found that CD71 (TFR1) expression was higher in KO RPMs (*Figure S13C*), and erythrophagocytosis was slightly increased in KO mice (*Figure S14*). According to our findings, LAMTOR2 KO RBCs expose more phosphatidyl serine (PS) (“eat-me” signal) (*Figure S14A*), than LMC RBCs, while CD47 remained unchanged (*Figure S14B*). Correspondingly, LAMTOR2 KO RMPs expressed more T-cell immunoglobulin and mucin domain-containing protein 4 (TIM4) (receptor for PS) (*Figure S14C*), whereas CD172a (receptor for CD47) remained unchanged (*Figure S14D*). Further, we studied the erythrophagocytosis capacity in BMDMs, and found that both LMC and LAMTOR2 KO engulf more LAMTOR2-originated RBCs than LMC-originated RBCs (*Figure S14F*). Surprisingly, phagocytosis of beads in vitro was rather reduced (*Figure S14E*) in LAMTOR2 KO BMDMs, indicating that stressed erythrocytes expressing more eat-me signals contributed to increased engulfment by macrophages expressing the specific PS uptake receptor TIM4.⁴⁰

When we analyzed iron metabolism proteins in BMDMs, we found that expression of TFR1 along with Tf uptake was reduced. We previously showed increased TFR1 expression in LAMTOR2 KO RPMs. Although BMDMs and RPMs are both macrophage subtypes, they differ substantially in their origin, specialized functions, originate from different culture conditions and microenvironment, which could explain the disparity.⁴¹ Of interest, recent evidence also suggested that specific tissue macrophages can affect transcellular iron shuttling by directly controlling the Tf/TFR1 mediated iron delivery to cells and the circulation.⁴²

When studying intracellular iron content and distribution in LAMTOR2 KO BMDMs in comparison to controls, we found neither differences in total iron content nor in the LIP levels, whereas more iron was detected in lysosomes. One explanation may be that iron intake is compensated by increased activity of DMT1, which is reflected by higher *Dmt1* mRNA expression in LAMTOR2 KO BMDMs (*Figure S15C*). On the other side,

Calcein-AM cannot cross membranes once hydrolyzed, and thus, cannot penetrate lysosomes and endosomes, which hold a big part of the LIP.⁴³ Hence, this assay might have underestimated the LIP in our setting. On the contrary, FerroOrange can detect free Fe²⁺ iron also in cellular compartments, and since more iron was found within lysosomes by LysoFerroRed, increased FerroOrange in KO BMDMs accounts for increased lysosomal iron in KO cells. It is likely then, that LAMTOR2 KO BMDMs have a diminished LIP, while free Fe²⁺ iron is retained in the endo/lysosomal system. Also, our RT-PCR data suggests that LAMTOR2 KO BMDMs are mobilizing more iron considering the increased nuclear receptor coactivator 4 (*Ncoa4*), *Steap3* and hypoxia-inducible factor 2 alpha (*Hif2α*) mRNA levels (*Figure S15D, F and G*).

We then focused on exploring the mechanism behind TFR1 downregulation in LAMTOR2 KO BMDMs, and whether its reduction was the result of decreased transcription/translation, or higher degradation. As shown in *Figure 6* and *Figure S9*, addition of BAF, but not BORT nor BI8622, increased TFR1. This increase was specific to the protein level, as mRNA remained unchanged. This suggests that TFR1 undergoes lysosomal degradation in LAMTOR2 KO BMDMs. TFR1 is known to be degraded both at the proteasome and at the lysosomes as a post-translational mechanism.^{24, 25} Both mechanisms are induced upon iron addition. In LAMTOR2 KO cells, endosomal traffic is abnormal and lysosomes are oddly expanded throughout the cytoplasm.³³ Thus, the proposed mechanism linking TFR1 degradation to the endolysosomal system is consistent with the existing literature and our own data. Consequently, we focused our subsequent investigation on lysosomes. First, we looked at lysosomal acidification. Previous literature has shown that LAMTOR5 deletion causes lysosomal de-acidification and blocked autophagic flux.⁴⁴ Meanwhile, deletion of LAMTOR1 leads to enhanced lysosomal function - more degradation.⁴⁵ As shown in *Figure 7*, lysosomes appeared more acidified in LAMTOR2 KO cells than in LMC, and acidification was blocked in both cases by BAF.

Other authors have shown that increased lysosomal acidification is linked to decreased mTORC1 activity by assembling V1 and V0 domains of the V-ATP to create the active proton pump.⁴⁶ Deletion of LAMTOR2 decreases mTORC1 activity,²⁹ thus, the increased lysosomal acidity could be explained by the decline of mTORC1 activity. In addition, Rag GTPases have also been involved in the regulation of lysosomal pH. GDP-bound RagA was observed to break the interaction between cytosolic chaperonin-containing tailless complex polypeptide 1 and V-ATPase, leading to the assembly of an active V-ATPase, which increases lysosomal acidification in *Drosophila melanogaster*.⁴⁷ Since, both LAMTOR1 and LAMTOR2 function as a link between V-ATPase and Rag GTPases,⁴⁷ is it plausible that deletion of LAMTOR2 leads to increased V-ATPase assembly and lysosomal acidification and degradation. In turn, a hyperacidified lysosome might degrade TFR1, which would explain the phenotype we observed.

In addition to being hyperacidic, lysosomes are also expanded throughout the cytoplasm in LAMTOR2 KO cells. Knowing that a correct distribution of the endolysosomal system is essential for properly transporting receptors, we wondered if TFR1 was being early missorted in the lysosomes, instead of the early endosomes and MVBs, exacerbating its degradation. For that, we conducted an experiment to evaluate the co-localization of TFR1 with the lysosomes using confocal microscopy. Interestingly, a significant increase of TFR1 co-localized with LAMP1 in BAF and BAF and FeN-treated samples as previously described.²⁵ As expected, upon addition of iron, more TFR1 was found closely localized to LAMP1 and no difference was observed between genotypes. However, upon addition of BAF and BAF and FeN, LAMTOR2 KO cells appeared to co-localize much better TFR1 and LAMP1,

suggesting that more TFR1 was trapped in lysosomal compartments unable to be degraded. These results are compatible with a model in which TFR1 ends up in the lysosomes more often and becomes degraded faster in their lower pH, speeding up TFR1 degradation.

Finally, we addressed whether any of the cellular changes we have observed could be the result of disrupted MAPK signaling. As pointed out, LAMTOR2 is involved in MAPK pathways by recruiting MEK1 on the late endosomes/lysosomes.³³ We have observed that phagocytosis of beads is reduced in LAMTOR2 KO BMDMs, which could be the result of altered MAPK signaling. Further, as previous research has found³¹, we showed that iNOS expression was strongly reduced, which might lead to impaired innate immune response due to decreased nitric oxide production. Whether this signaling alteration is also involved in the iron phenotype remains to be determined in future research.

In summary, this study is the first to establish a critical role for LAMTOR2 in regulating systemic and cellular iron metabolism. Our findings also open up several avenues for future research. In particular, the direct mechanistic link between LAMTOR2-deficient macrophages and the broad spectrum of iron dysregulation observed requires further elucidation.

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Figure legends

Figure 1. Blood, serum and organ analysis in LysMCre (LMC) and LAMTOR2 KO mice.

A) Reticulocyte percentage was measured by flow cytometry. B) Spleen weight was normalized to mouse weight. C) Serum hepcidin was measured by enzyme-linked immunosorbent assay (ELISA). D) Liver *Hamp* mRNA levels. E) Serum erythropoietin (EPO) was also measured by ELISA. F) Serum iron measurement. Unpaired T-test was applied for panel E and Welch's test was used for panels A, B, C, D and F. *P<0.05, **P<0.01, ***P>0.001, ****P<0.0001. Results are shown as mean ± standard deviation. 18 LysMCre and 16 LAMTOR2 KO mice were analyzed. Results are from two and three independent experiments.

Figure 2. Organ tissue iron quantification.

A to D) Colorimetric quantification of total iron in tissue sample homogenates from liver, spleen and duodenum and bone marrow (BM). E) Representative Prussian Blue staining images from spleen, BM and liver. Blue corresponds to iron staining (black arrows). Scale bar for spleen images is 50 µm. For the rest of the images, scale bar is 20 µm. Mann Whitney test was used in panel A, C and D. Unpaired T-test was employed in panel B. Welch's test was applied for panel F and I. *P<0.05, **P<0.01, ***P>0.001, ****P<0.0001. 18 LysMCre (LMC) and 16 LAMTOR2 KO mice were analyzed. Results are shown as mean ± standard deviation. Results are from three independent experiments.

Figure 3. Western blot (WB) of transferrin receptor 1 (TFR1), ferroportin 1 (FPN1) and ferritin (FT) liver, spleen and bone marrow (BM) alongside flow cytometry or erythroid progenitors in the BM and spleen.

A) Representative western blots of liver, spleen and bone marrow (BM), where transferrin receptor 1 (TFR1), ferroportin 1 (FPN1), ferritin (FT) were analyzed. β -Actin (ACTB) was used as the housekeeping gene. B Flow cytometry analysis of erythroid progenitors in the BM. Basophilic, polychromatic and orthochromatic erythroblasts are represented. Unpaired T-test was used for the statistical analysis. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Results are shown as mean ± standard deviation. Results are from three independent experiments were 13 LysMCre (LMC) and 13 LAMTOR2 KO mice were analyzed.

Figure 4. Transferrin receptor 1 (TFR1) is downregulated in LAMTOR2 KO bone marrow-derived macrophages (BMDMs) along with transferrin (Tf) uptake.

A) Western blot of BMDMs in control (Ctrl) conditions and after applying ferric nitrate nonahydrate (FeN) for 8 and 24 h. TFR1, ferroportin 1 (FPN1) and ferritin (FT) were analyzed, using β -Actin (ACTB) as the housekeeping protein. The blot shown is a representative cropped image; non-relevant lanes were removed. All samples were run on the same gel but were non-contiguous; intervening lanes were removed and are indicated by dividing lines. B) Quantification of Tf fluorescence intensity by immunofluorescence microscopy. Cells were incubated for 20 min with Tf-AF488 and green fluorescence intensity signal was measured in every cell. C) Representative immunofluorescence image showing cellular uptake of Tf. Tf-AF488 is shown in green

and DAPI, indicating the nuclei, in blue. Scale bar is at 10 μ m. D and E) Tf uptake by flow cytometry. D) Shows the quantification of Tf uptake represented as mean fluorescence intensity (MFI). E) Representative histogram from FlowJo illustrating Tf uptake profiles. LAMTOR2 KO, represented in blue, displays a left-shifted peak, compare to LysMCre (LMC) (in orange) indicating reduced Tf internalization. Welch's test was applied in panel B. 2-way ANOVA with Tukey's multiple comparison was applied in panel D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. In panel B, 4000 cells were analyzed. In panel D, two independent experiments were performed in duplicates. Each dot represents one technical replicate. Results are shown as mean $\pm$ standard deviation.

Figure 5. Quantification of intracellular iron in LAMTOR2 KO bone marrow-derived macrophages (BMDMs).

A) Atomic absorption spectrometry (AAS) of BMDMs in control (Ctrl) conditions and after applying ferric nitrate nonahydrate (FeN) for 4 and 24 h. This assay measured the total amount of intracellular iron. B) Calcein-AM assay was used to measure the labile iron pool (LIP) of BMDMs in Ctrl conditions and after applying FeN for 4 and 24 h. Calcein-AM was used in consonance with the iron chelator deferoxamine (DFO) and the LIP was calculated as the difference between Calcein-AM fluorescence intensity and after applying DFO. C) FerroOrange was employed to quantify intracellular free ferrous Fe^{2+} iron using live-cell fluorescent microscopy. For each cell, the integrated fluorescence intensity (IFI) of FerroOrange-positive objects was normalized to their cumulative object area. D) LysoFerroRed quantification was used to measure free Fe^{2+} lysosomal iron using live-cell fluorescent microscopy. For each cell, the integrated IFI of LysoFerroRed-positive objects was normalized to their cumulative object area. Data are represented as fold change relative to LysMCre (LMC). E) Representative images of LysoFerroRed-stained LMC and LAMTOR2 KO BMDMs. LysoFerroRed is represented in yellow, while DAPI is represented in blue. Scale bar is 10 μ m. 2-way ANOVA with Tukey's multiple comparison was applied in panels A and B. Welch's test was applied in panel C, and Unpaired T-test was applied in panel D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation. Results are from two and three independent experiments performed in duplicates or triplicates. Each dot represents one technical replicate.

Figure 6. Transferrin receptor 1 (TFR1) is rescued by bafilomycin (BAF) but not by bortezomib (BORT).

A) Western blot (WB) of bone marrow-derived macrophages (BMDMs) in control (Ctrl) conditions and after applying 50 nM of BORT for 12 h. TFR1 and the housekeeping gene β -Actin (ACTB) were analyzed. B) Densitometric quantification from panel A. C) WB of BMDMs in Ctrl and after applying 10 nM of BAF for 12 and 24 h. TFR1 and the housekeeping gene ACTB were analyzed. D) Densitometric quantification from panel C. 2-way ANOVA with Sidak's multiple comparison test was applied in panel B. 2-way ANOVA with Tukey's multiple comparison test was applied for panel D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation. Results are from two and three independent experiments performed in duplicates. Each dot represents one technical replicate.

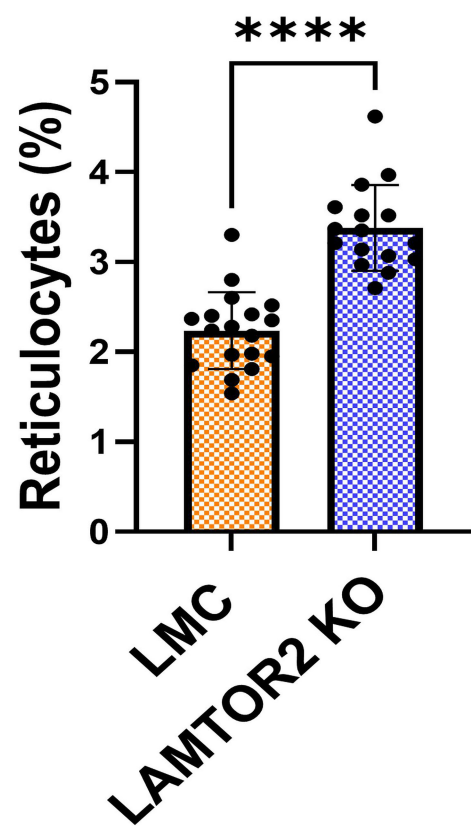
Figure 7. LAMTOR2 knockout (KO) bone marrow-derived macrophages (BMDMs) show hyperacidified lysosomes in comparison to LysMCre (LMC).

A) Representative live-cell fluorescence microscopy images. Lysosomal Acidic pH Detection Kit from Dojindo was used. In green, Lyso Prime stains the lysosomes while pH Lys is used to monitor lysosomal acidification. Bafilomycin (BAF) was employed as a negative control. B) Graph showing the normalized fluorescence intensity of pH Lys Red relative to Lyso Prime Green. Fluorescence was measured on a per-cell basis and around 200 cells per condition were analyzed. 2-way ANOVA with Tukey's multiple comparison test was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation. Results are from three independent experiments, were 200 cells per group were analyzed. Scale bar is 10 μm . Ctrl = Control.

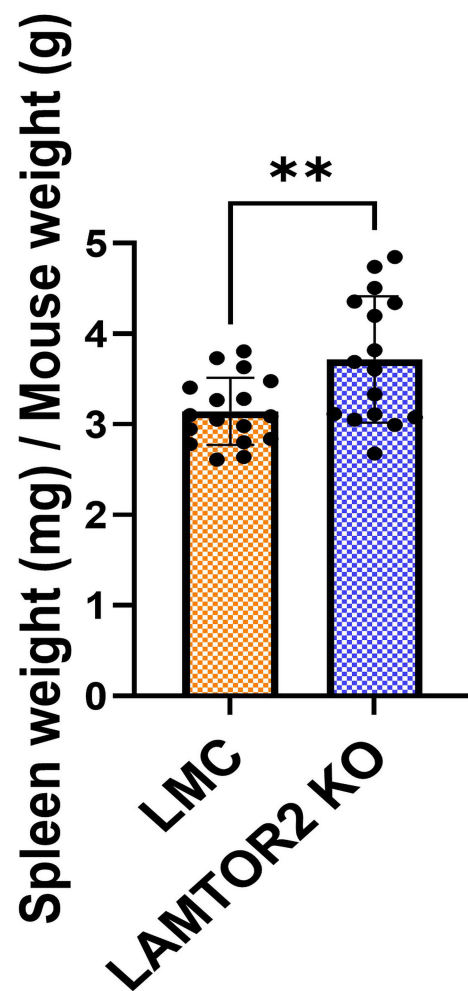
Figure 8. Transferrin receptor 1 (TFR1) colocalizes better with lysosome associated membrane protein 1 (LAMP1) in bafilomycin (BAF)-treated LAMTOR2 knockout (KO) bone marrow-derived macrophages (BMDMs) in comparison to LysMCre (LMC).

A) Representative confocal images of LMC and LAMTOR2 KO BMDMs treated for 12 h with ferric nitrate nonahydrate (FeN), BAF, FeN +BAF and control (Ctrl). DAPI is represented in blue, LAMP1 in green and TFR1 in red. Merged images are on the right side. B) Graph showing Person's coefficient between TFR1 and LAMP1 across the four different conditions. 2-way ANOVA with Tukey's multiple comparison test was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation. Results are from three independent experiments with a total of 10 to 13 cells analyzed per group. Scale bar is 10 μm .

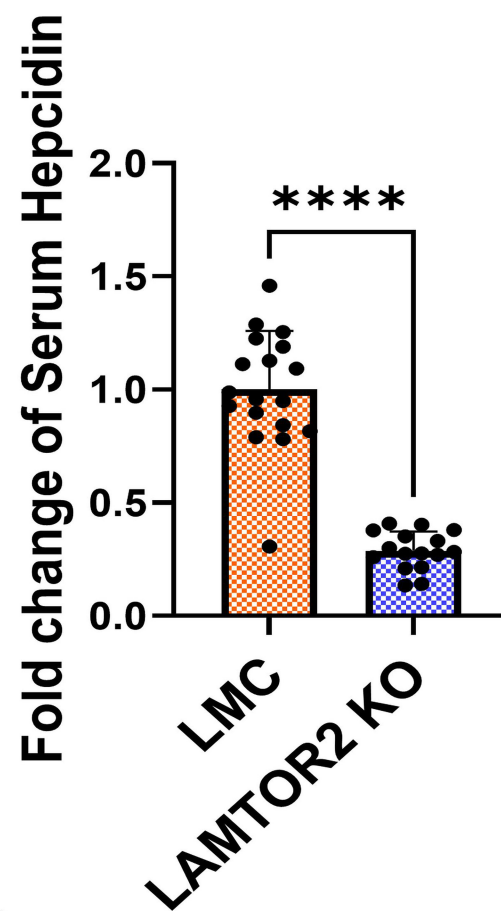
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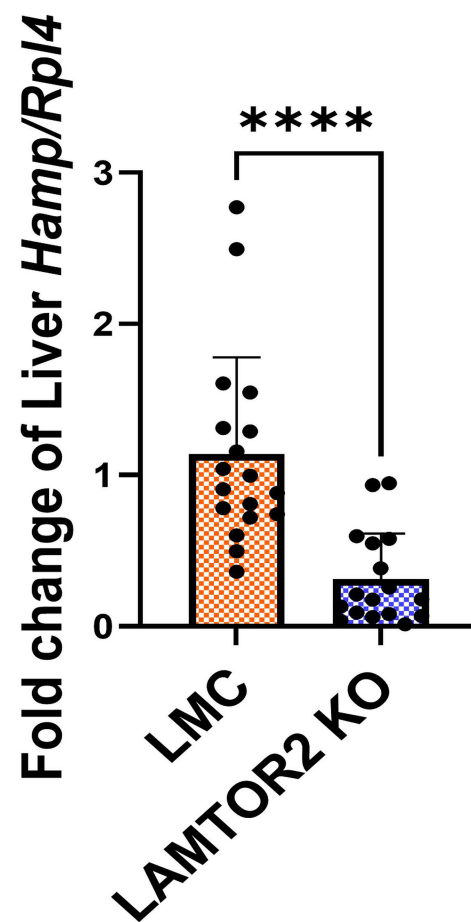
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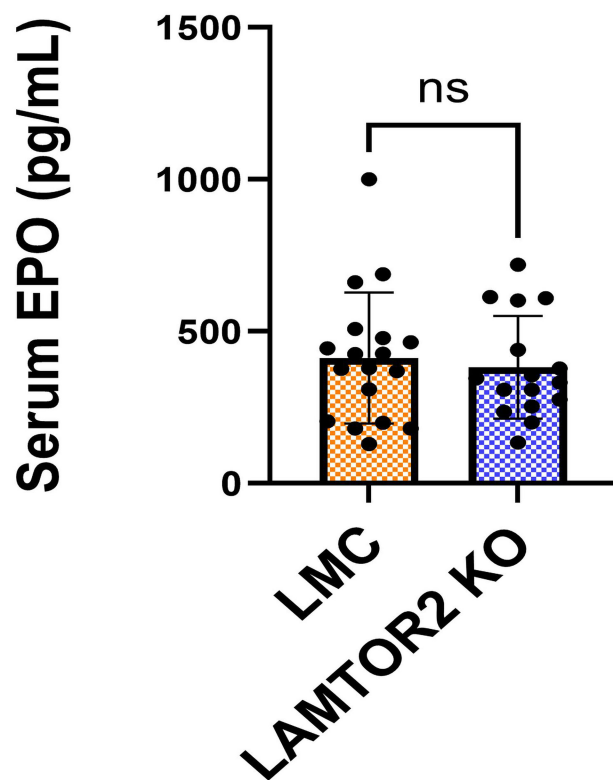
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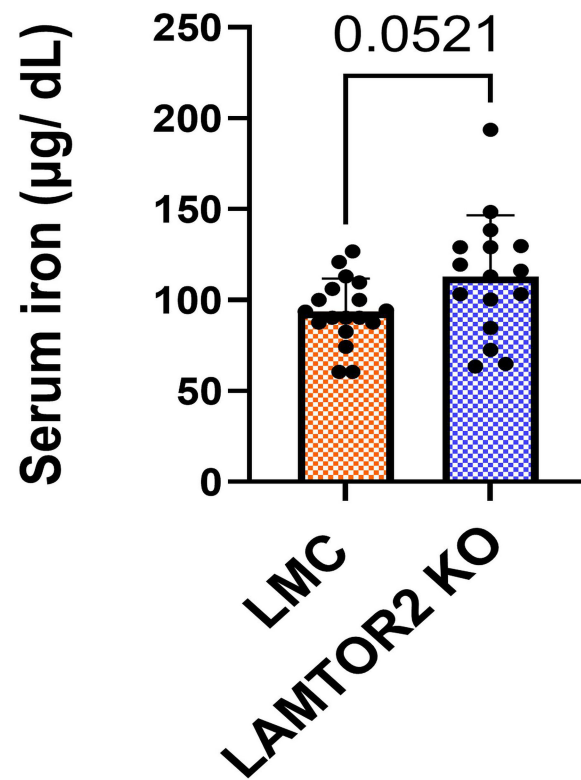
D)



E)

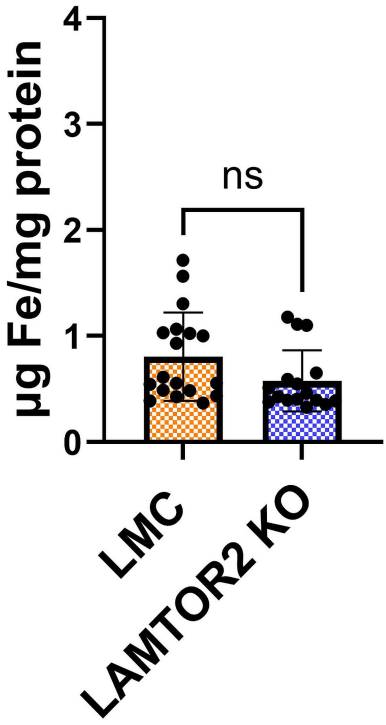


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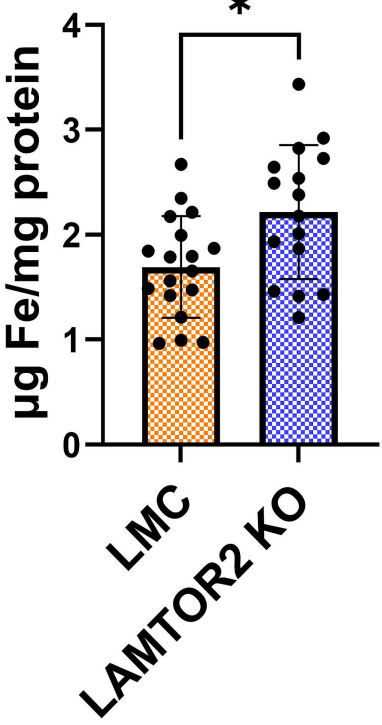
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Liver



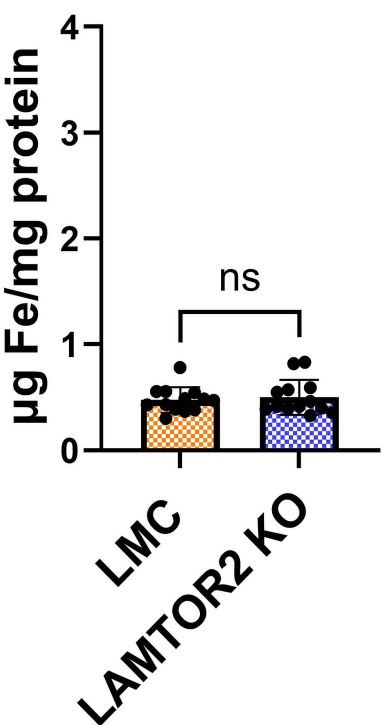
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Spleen



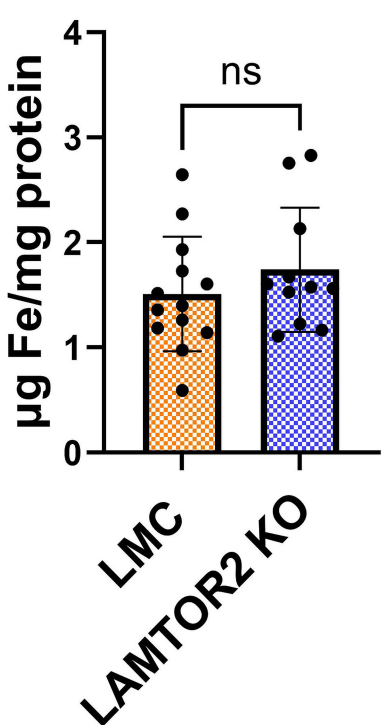
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Duodenum



D)

BM

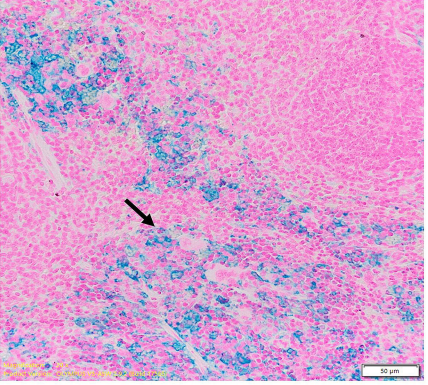
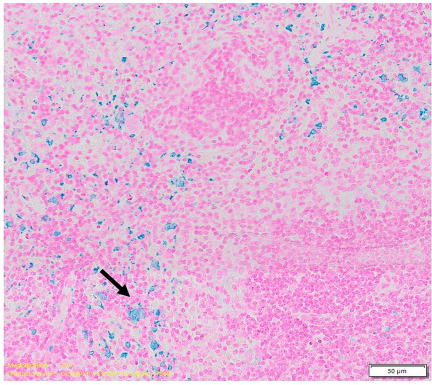


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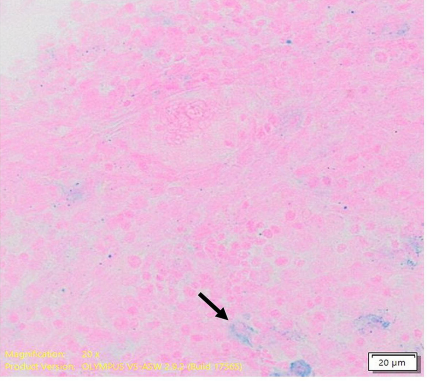
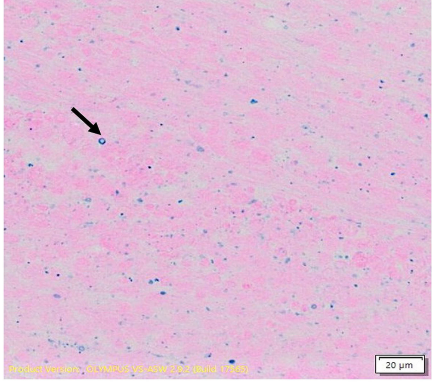
LMC

LAMTOR2 KO

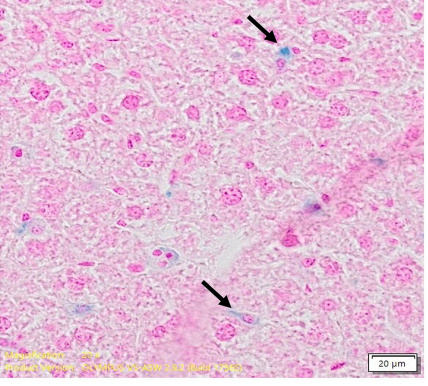
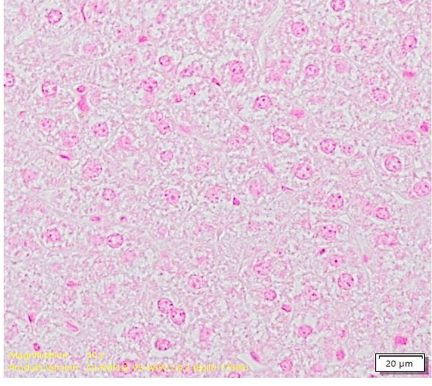
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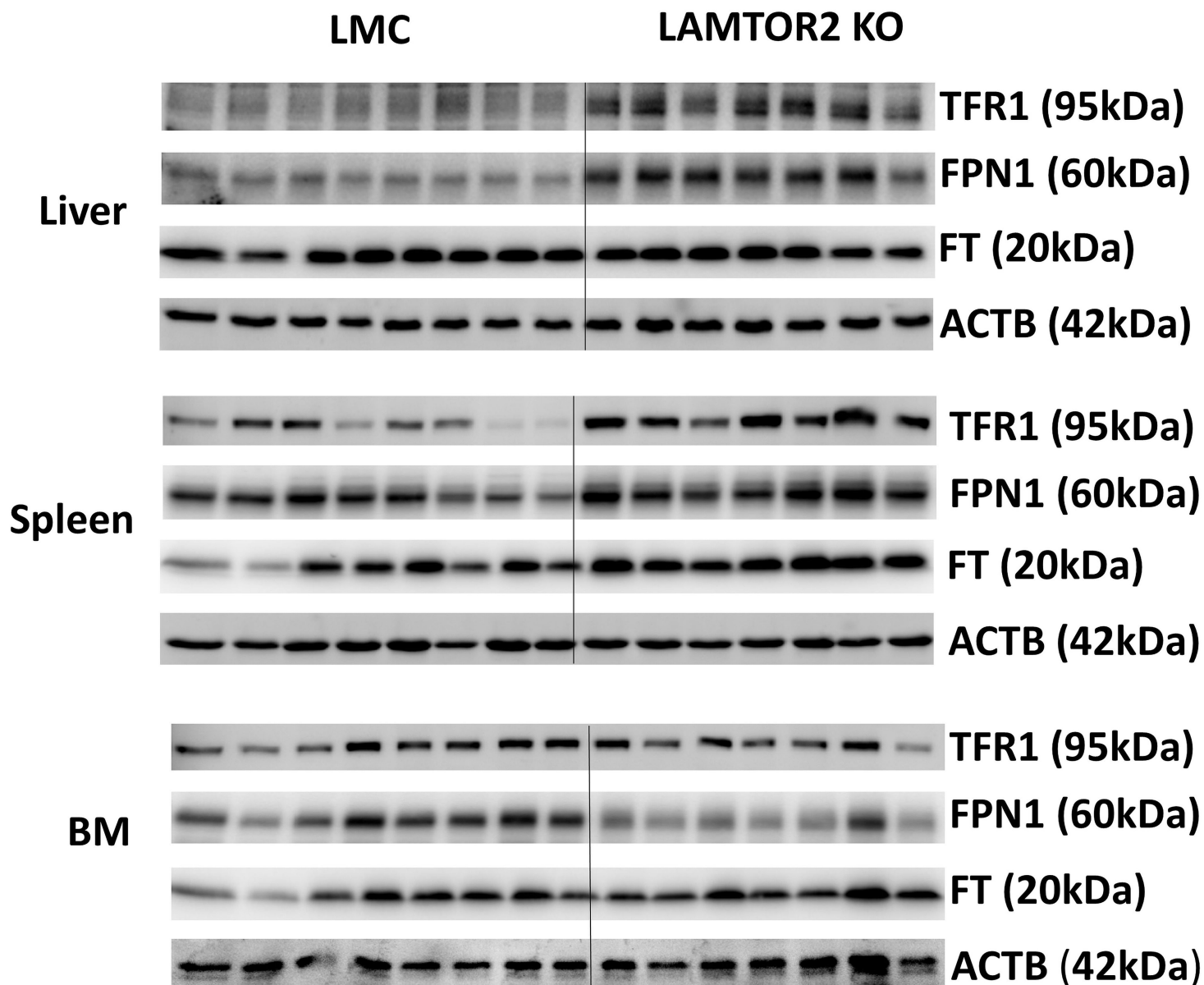
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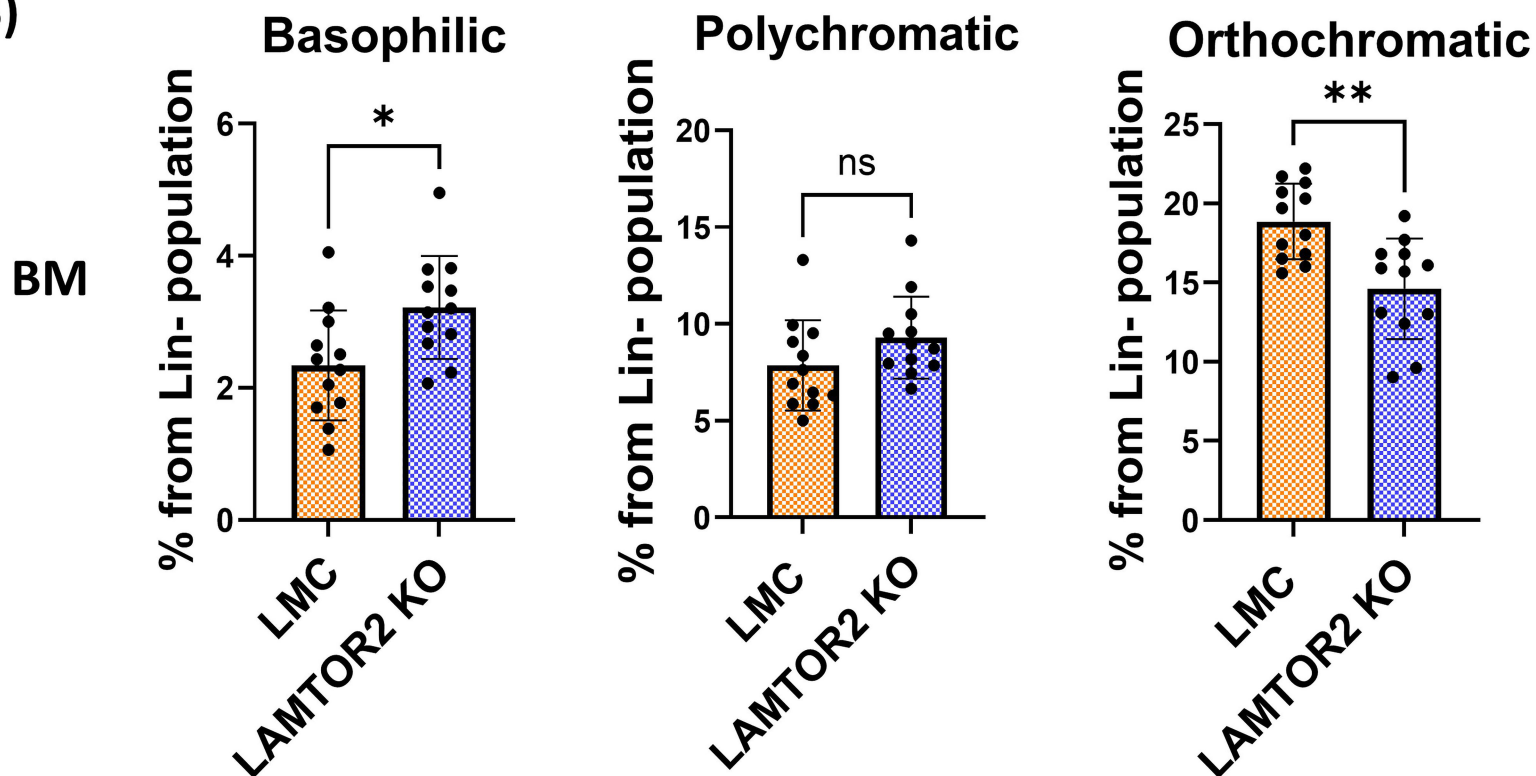
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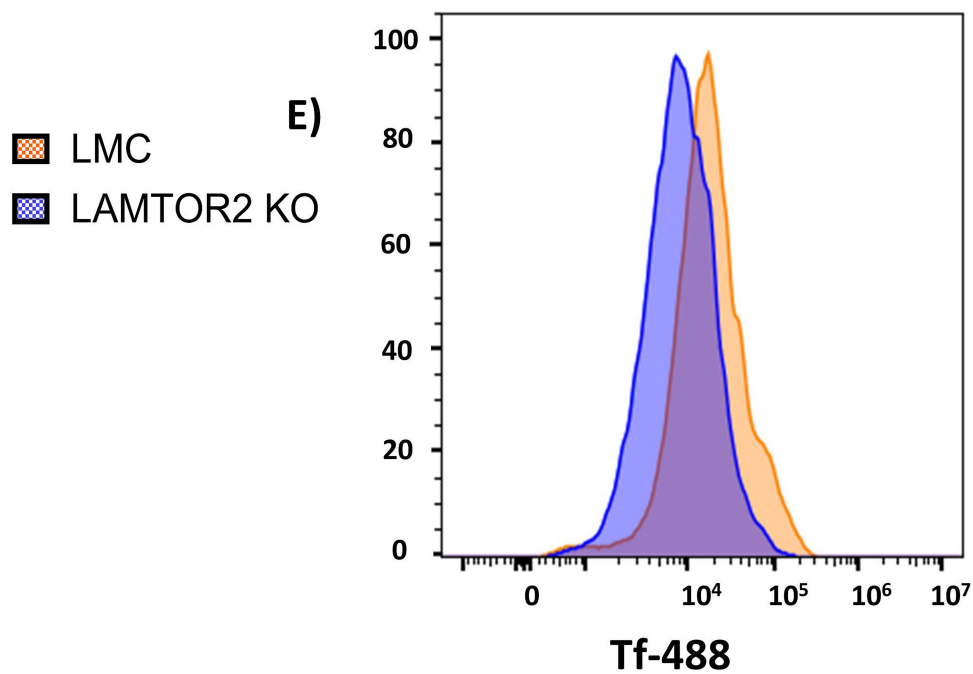
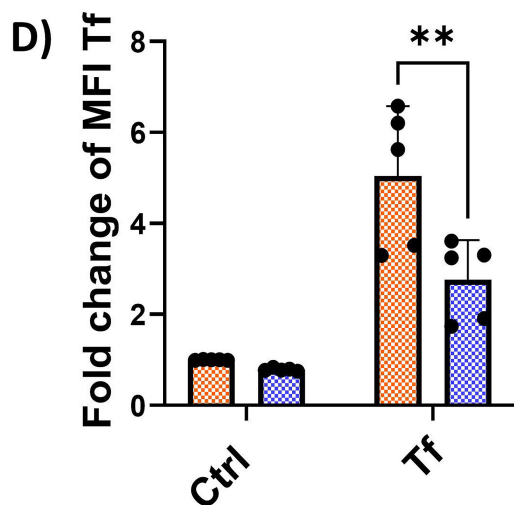
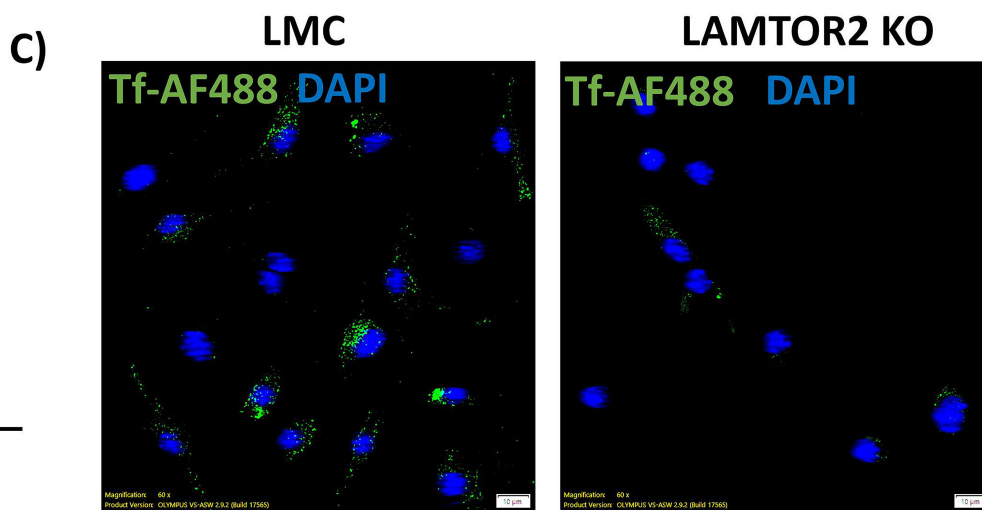
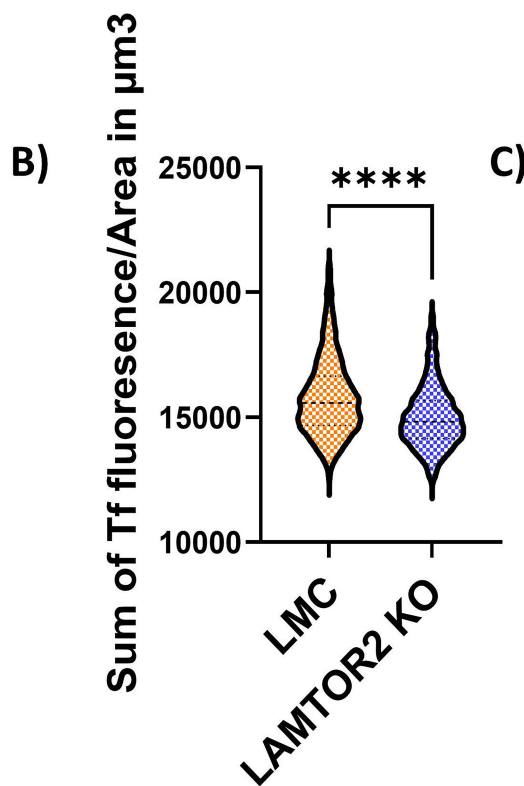
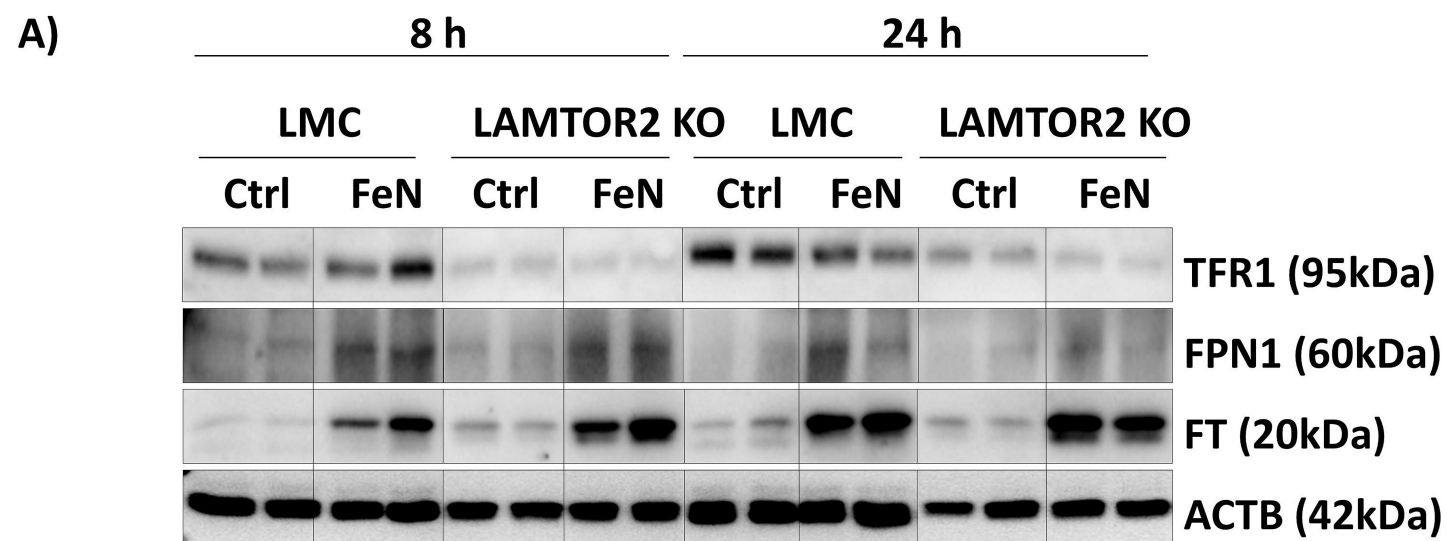


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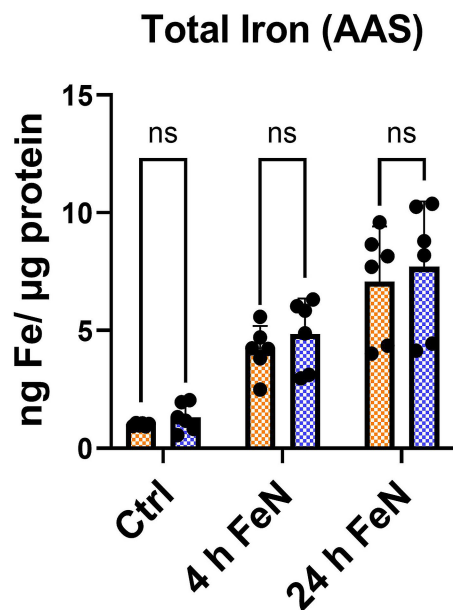


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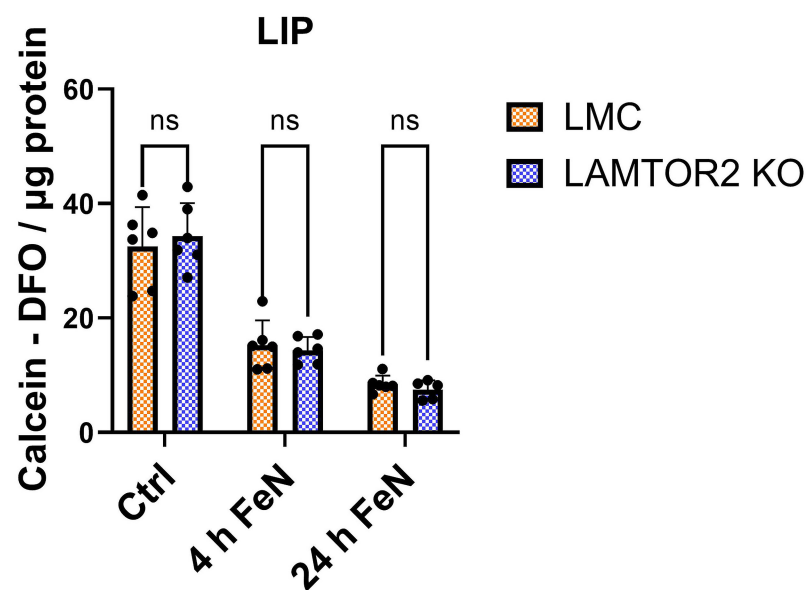




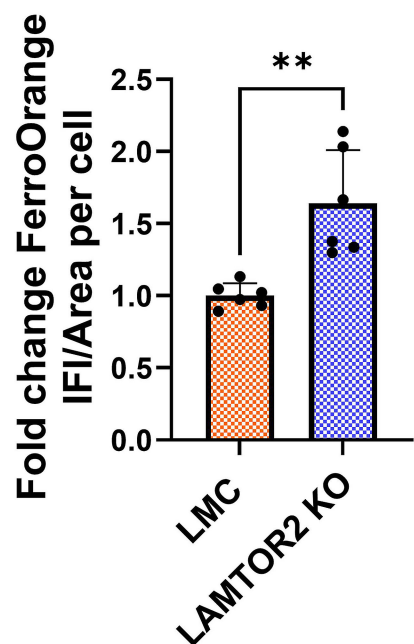
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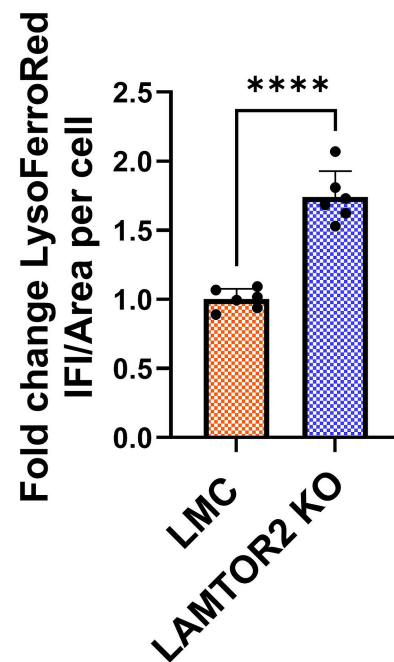
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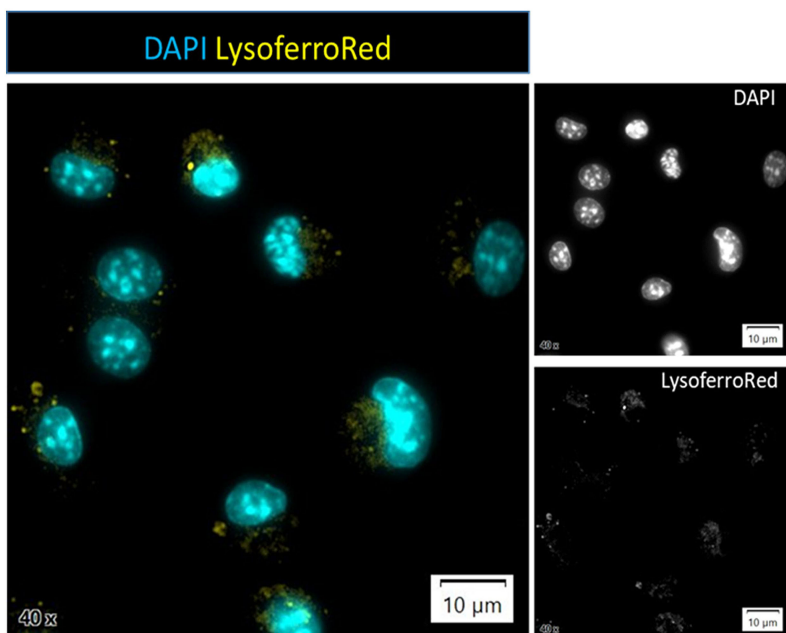


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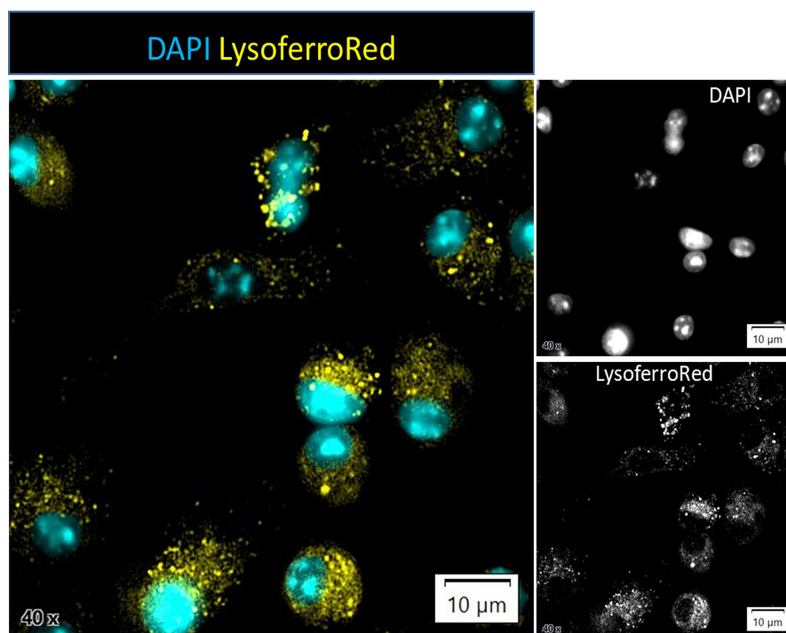


E)

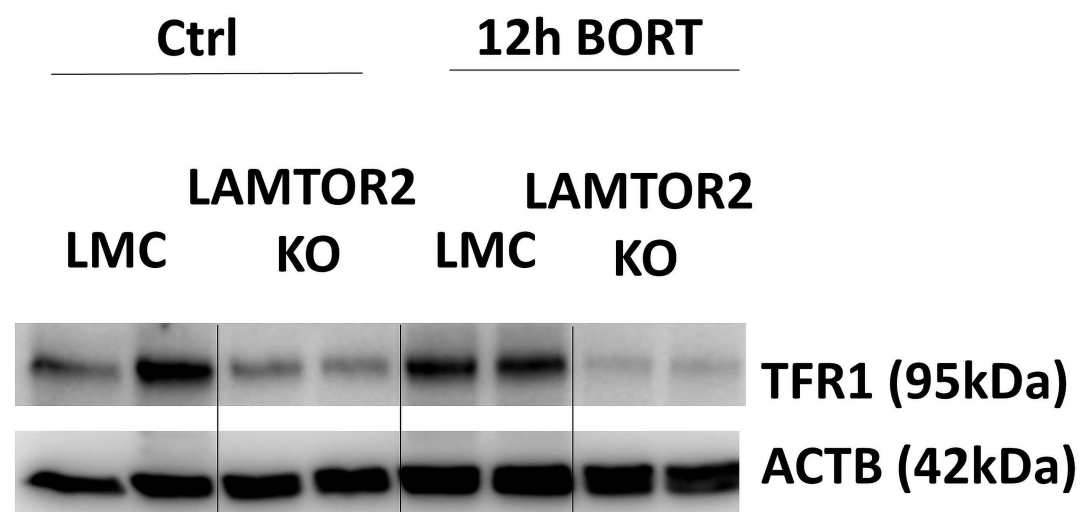
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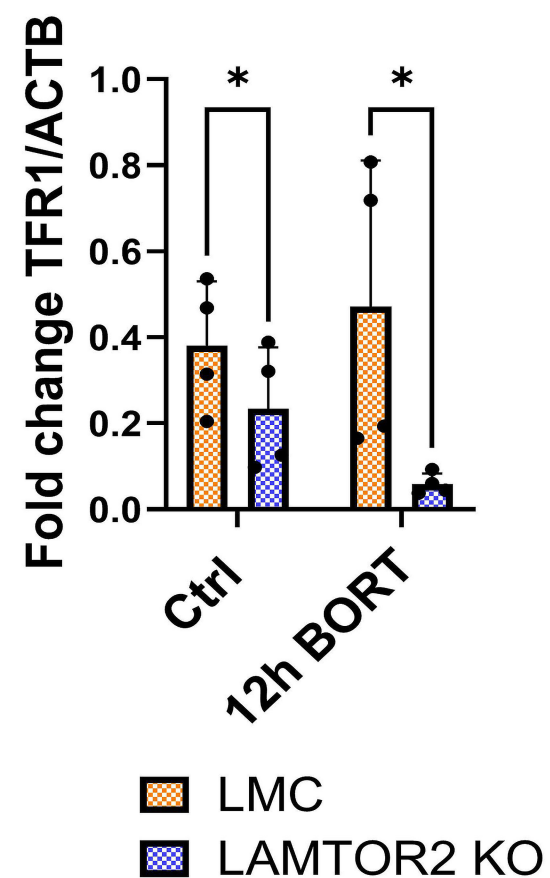
LAMTOR2 KO



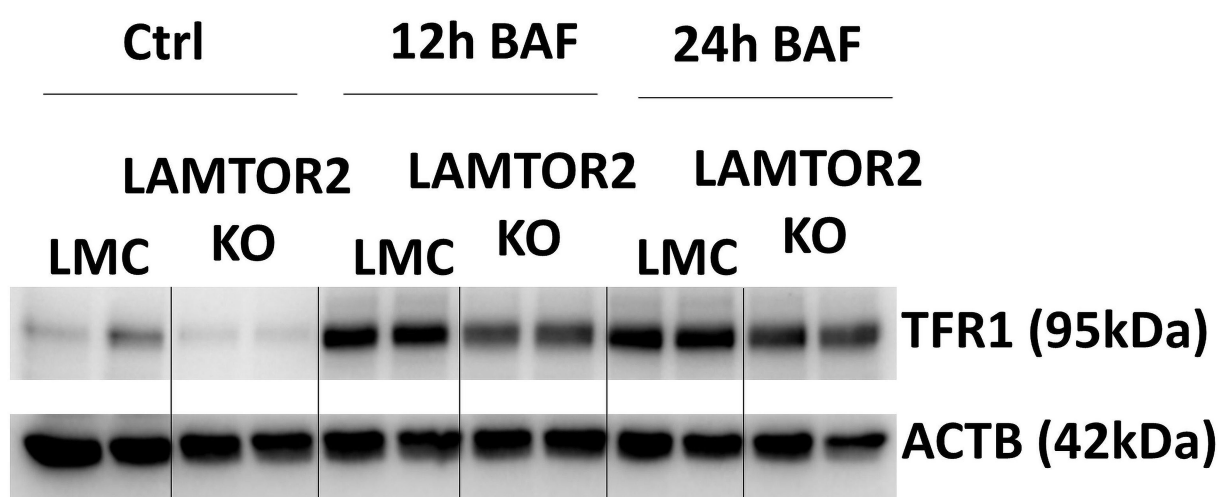
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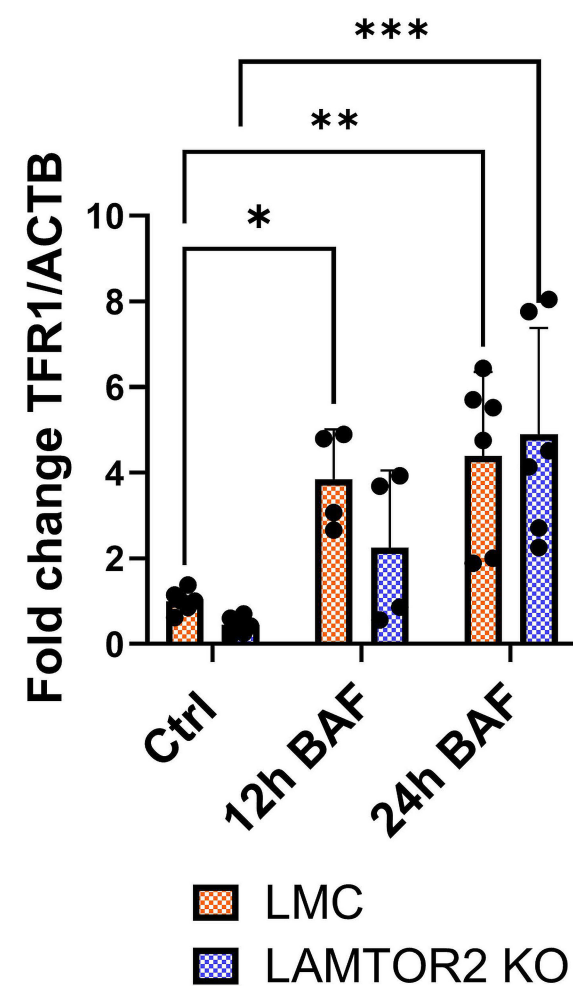
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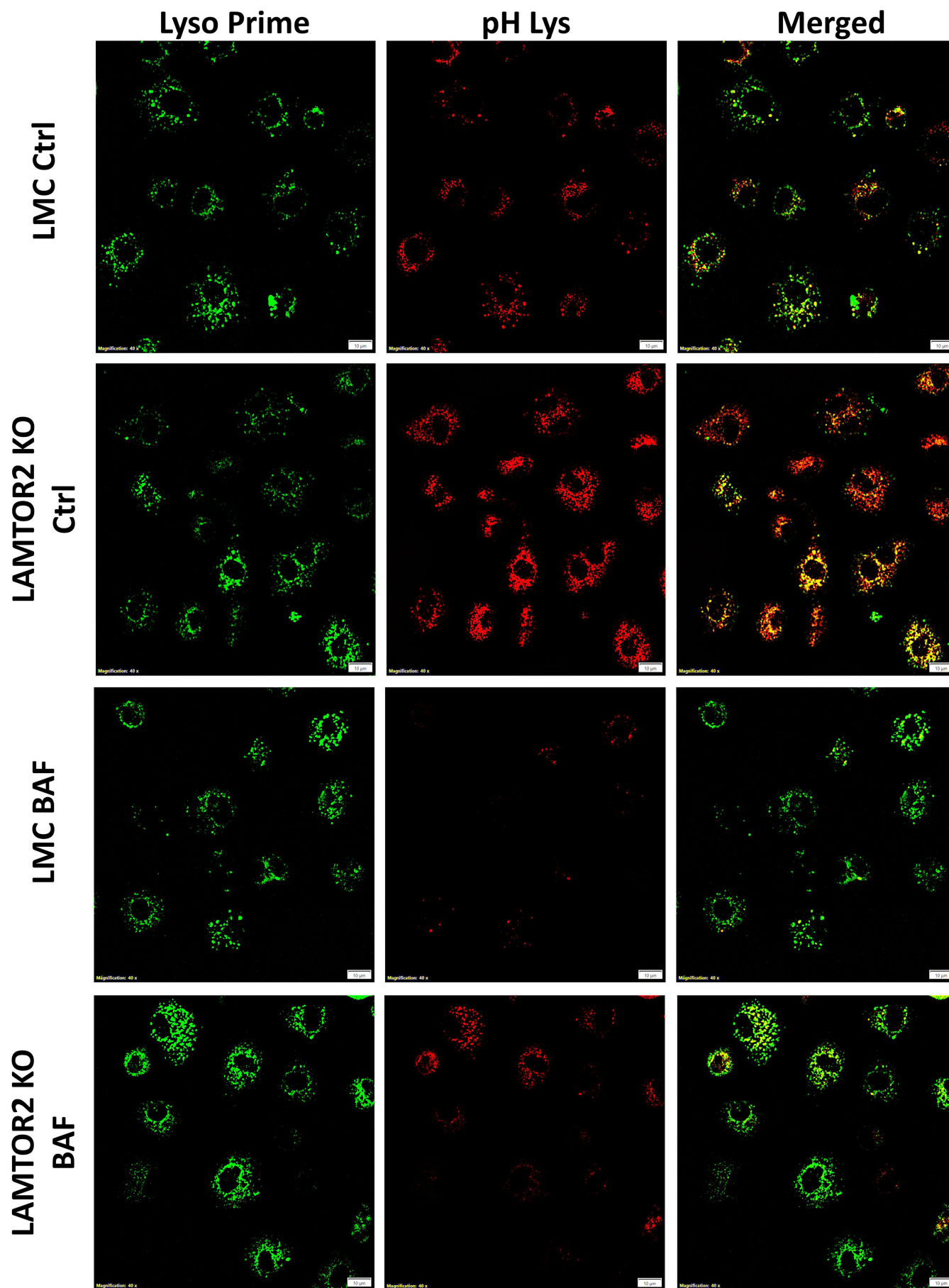
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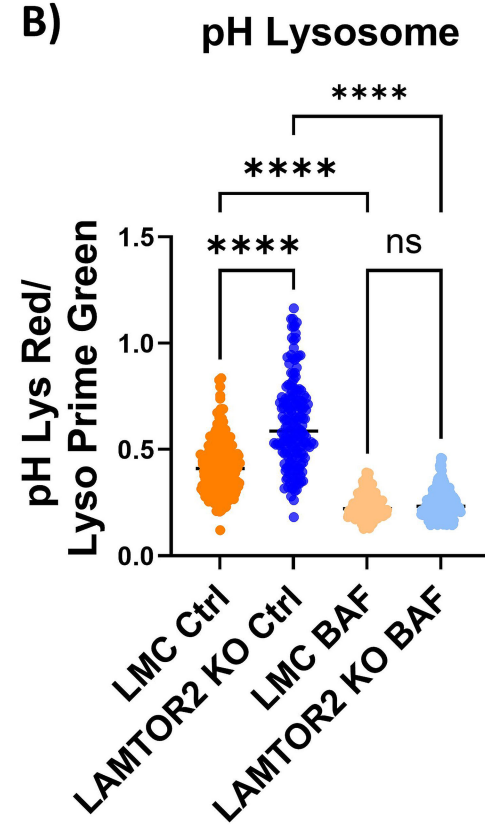
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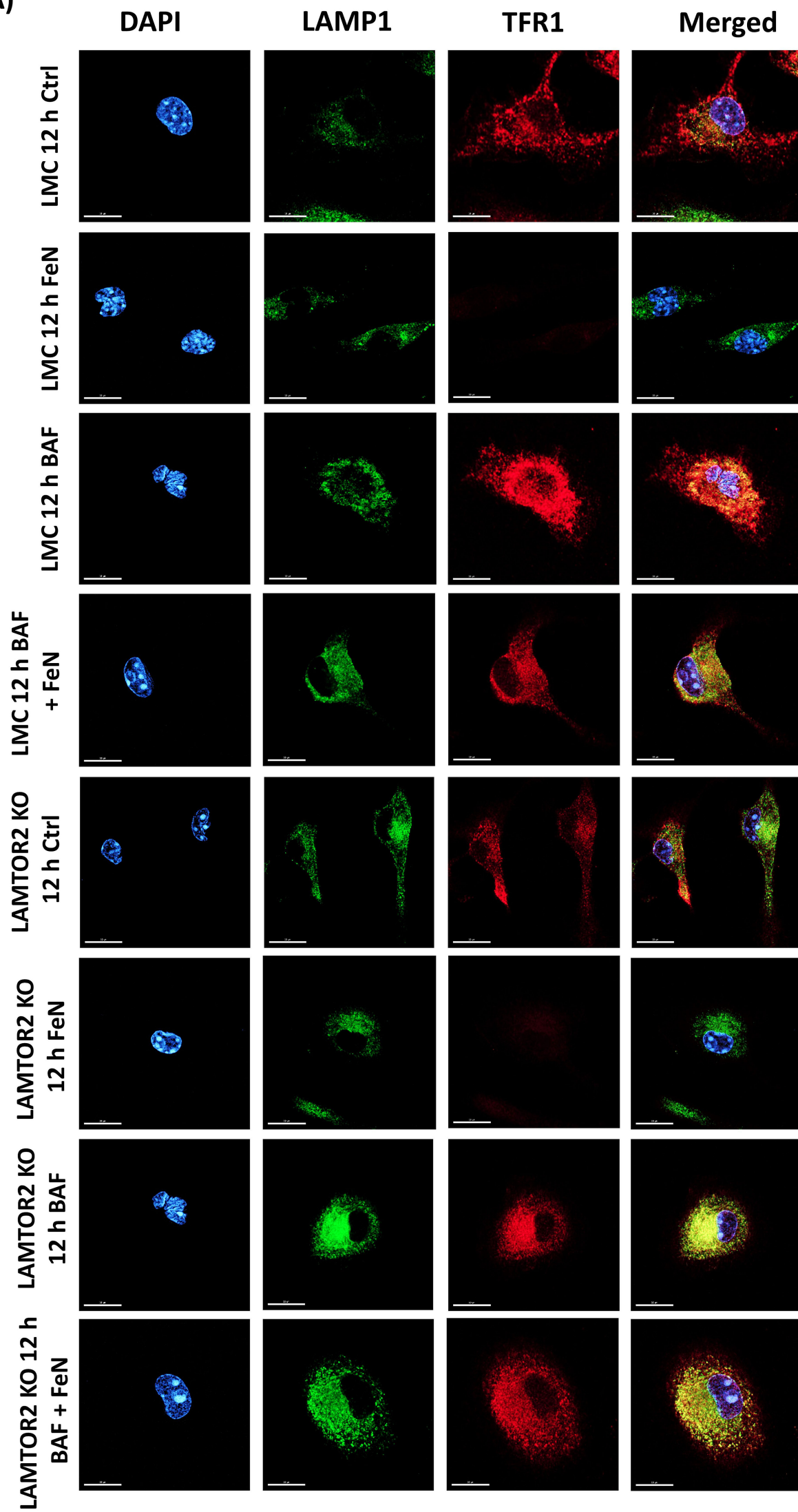
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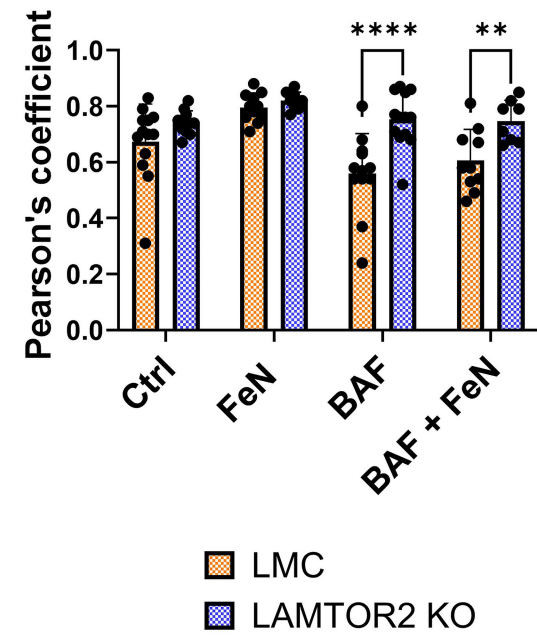
B)



A)



B)



Supplementary methods

Generation of LAMTOR2^{fl/fl} mice and myeloid-specific LAMTOR2^{-/-} mice

LAMTOR2^{fl/fl} mice were produced by Teis, Taub ¹. Briefly, the targeting construct with exons 1 to 4 flanked by *loxP* sites, PGK-Neo and *frt* sites was transfected into G418-resistant HM1 embryonic stem cells (ESC), replacing one LAMTOR2 allele, and successful clones were identified by Southern blotting. Two independent ESC clones with the targeted allele were injected into blastocysts of C57BL/6, and the generated chimeric mice were crossed with C57BL/6 to obtain a germline transmission of the targeted allele (LAMTOR2^{targ/+}). LAMTOR2^{targ/targ} mice were crossed with hACTB::*Flpe* mice, which deleted the *frt*-flanked PGK-Neo selection cassette and retained the *loxP* sites, to generate LAMTOR2^{fl/+} mice, which was confirmed by PCR analysis. Conditional deletion of LAMTOR2 in lysozyme-M-positive cells was achieved by crossing LAMTOR2^{fl/fl} mice with LysMCre mice. The resulting mice were further bred until LysMCre LAMTOR2^{-/-} mice were obtained.

Reticulocyte count

To quantify the percentage of reticulocytes in mouse blood, 1 μ L of heparinized blood was diluted in 50 μ L of FACS buffer and centrifuged at maximum speed for 10 s. After removing the supernatant, cells were incubated with antibodies Ter119-APC/eFluor 780 (1:200; eBioscience, San Diego, CA, USA), CD45-Pacific Orange (1:800; Invitrogen, Carlsbad, CA, USA) and CD71-eFluor 450 (1:200; Invitrogen) diluted in FACS buffer for 15 min at room temperature (RT) in the dark. Cells were then centrifuged and washed once and were finally analyzed with CytoFlex STM Flow cytometer (Beckman Coulter, Brea, CA, USA). Thiazole orange was also used (1:10,000) to confirm the RNA-positive reticulocytes and yielded comparable results. For the gating strategy, debris was excluded with FSC-A vs SSC-A gate, followed by identification of singlets which were identified by FSC-A vs FSC-H. Subsequently, CD45-negative gating was applied to exclude leukocytes, and from the remaining population, reticulocytes were identified by gating for CD71⁺/Ter119⁺ cells. Data were analyzed using FlowJo 10.7.0 software (BD Biosciences, Ashland, OR, USA).

Perls' Prussian Blue Staining

Liver, spleen and duodenum sections were fixed in formalin and embedded in paraffin. Sections (5 μ m) were treated with 20% ammonium sulfide solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 min, rinsed under running tap water for 10 s, and subsequently incubated with 10% potassium hexacyanoferrate (Carl Roth, Karlsruhe, Germany) mixed with 0.5 M hydrochloric acid (Sigma-Aldrich) for another minute. After another 10 s of washing with tap water, nuclei were counterstained with nuclear fast red (Gatt-Koller, Absam, Austria) for 5 min. Sections were washed again and then dehydrated sequentially in 100% acetone (10 min at 4 °C), 96% ethanol (2 min), and absolute ethanol. Subsequently, sections were cleared in xylene for 2 min and mounted with Entellan mounting medium (Sigma-Aldrich). Images were obtained using an Olympus BX61VS slide scanner (Olympus, Tokyo, Japan) equipped with a 20x/0.75 NA objective and analyzed with OlyVIA software (Olympus).

Erythroid progenitor analysis

Bone marrow (BM) cells were isolated from mouse femurs by centrifugation. The resulting pellet was resuspended with Dulbecco's phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria) and passed through a 40 μ m sieve. Subsequently, the cell suspension was stained with biotin-conjugated antibodies against CD19 (1:200; BioLegend, San Diego, CA, USA), CD3 (1:200; BioLegend), CD49b (1:200; BioLegend), CD11c (1:200; BioLegend), CD11b (1:200; BioLegend) and Gr1 (1:200; BioLegend) which were used to identify lineage-positive cells. Secondary labelling was performed with Streptavidin-PE-e1610 (1:200; eBioscience). In addition, cells were stained with CD44-APC (1:200; BioLegend) to assess erythroid maturation.

Flow cytometric analysis was performed using the following gating strategy: FSC-A vs SSC-A to exclude debris; FSC-A vs FSC-H for singlets; Streptavidin-PE-eFluor 610 vs FSC-A to exclude lineage-positive hematopoietic cells and identify the lineage-negative (Lin⁻) progenitor population, and CD44-APC against FSC-A to distinguish the four different erythroid progenitor populations. Based on CD44 and FSC-A, four populations were identified: CD44 low corresponded to red blood cells (RBCs) (I), CD44 intermediate was gated as orthochromatic erythroblasts (II), CD44 intermediate corresponded to polychromatic erythroblasts (III) and CD44 high was gated as basophilic erythroblasts (IV).

Spleen cells were obtained by mashing the organ through a 100 μ m cell strainer with PBS and the same staining and gating strategy used for the BM was applied.

Production and seeding of bone marrow-derived macrophages (BMDMs)

8 to 12-week-old mice were euthanized and their BM was collected from femurs and tibias. The bones were placed in microcentrifuge tubes and centrifuged for 3 s at maximum speed. The pellet was resuspended in PBS supplemented with Penicillin-Streptomycin (P/S, PAA Laboratories), transferred to a Falcon tube and centrifuged at 1250 rpm for 5 min at 4 °C. After removing the supernatant, the pellet was incubated for 3 min with 2 mL of Ery-Lysis buffer (1:10; R&D, Minneapolis, MN, USA) to lyse the erythrocytes, then washed with 15 mL of PBS supplemented with P/S and centrifuged at 4 °C at 1250 rpm for another 5 min. Two more rounds of washing and centrifugation followed until the final pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Basel, Switzerland), supplemented with 10% fetal calf serum (FCS; Biochrome AG, Berlin, Germany), 2 mmol/L L-glutamine (PAA Laboratories), 1% P/S, and seeded on 15-cm dishes. Macrophage colony-stimulating factor (M-CSF; 50 ng/mL; PeproTech, Rocky Hill, NJ, USA) was added to promote the differentiation and proliferation of macrophages. The cells were incubated at 37 °C in a humidified atmosphere with 5% carbon dioxide for 6 days, with the medium refreshed every other day. Upon cell differentiation, macrophages were washed twice with PBS + P/S, gently scraped (Greiner, Kremsmünster, Austria), centrifuged and resuspended for cell counting with DMEM supplemented with 1% FCS, 2 mmol/L L-glutamine and 1% P/S. Cells were counted with EVETM Automated Cell Counter (NanoEnTek, Seoul, South Korea).

For RNA and plate reader analysis, 700,000 cells were seeded in 6-well plates (Falcon, Corning, NY, USA); for flow cytometry, 1,000,000 cells were seeded also in 6-well plates; for protein analysis and atomic absorption spectrometry (AAS), 2,000,000 cells were seeded in 22 cm² dishes (TPP, Trasadingen, Switzerland). For fixed-cells microscopy, 80,000 cells were seeded in 24-well plates (Falcon) with coverslips; and for live-cell microscopy, 80,000 cells were seeded in μ -Slide 8-well high chamber slides (Ibidi, Gräfelfing, Germany). As treatments, bafilomycin A1 (BAF; 10 nM; MCE,

Monmouth Junction, NJ, USA), iron (III) nitrate nonahydrate (FeN; 25 μ M; Sigma-Aldrich), BI8622 (5 μ M; MCE) and Bortezomib (50 nM; MCE) were used for periods ranging from 4 to 24 h.

Protein analysis

Protein extracts from BMDMs and organ homogenates were obtained using a cytoplasmic lysis buffer (25 mM Tris-HCl pH 7.4, 40 mM KCl, 1% Triton X-100). The protein concentration was determined with the Bradford reagent (Bio-Rad, Hercules, CA, USA) and an amount of 15–20 μ g was loaded onto 10% SDS-polyacrylamide gels. Electrophoresis was performed at 180 V for approximately one hour to separate the proteins, which were then transferred onto a PVDF membrane (GE Healthcare, Chicago, IL, USA) at 100 V. To visualize the protein bands, Ponceau staining was applied to visualize proteins and cut the membrane into strips at the appropriate molecular weights. To minimize nonspecific antibody binding, the membrane was blocked with 5% milk powder. Primary and secondary antibodies were introduced sequentially, with multiple washing steps using TTPS in between. Protein detection was carried out through horseradish peroxidase-conjugated antibodies (1:2000; Bio-Rad; anti-rabbit), (1:4000; Dako; anti-mouse). For quantification, densitometry data were acquired on the ChemiDoc Touch Imaging System (Bio-Rad) and analyzed using the Image Lab software (Bio-Rad). The antibodies used included a mouse anti-transferrin receptor 1 (TFR1) antibody (1:1000; Sigma-Aldrich; MW: 100 kDa), a rabbit anti-ferritin (FT) antibody (1:500; Sigma-Aldrich; MW: 20 kDa), a rabbit anti-ferroportin 1 (FPN1) antibody (1:2000; Eurogentec, Seraing, Belgium; MW: 66 kDa) and a rabbit anti- β -actin antibody (ACTB) (1:500; Sigma-Aldrich; MW: 42 kDa).

For hepcidin signaling, we used rabbit anti-ERK 1/2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-pERK 1/2 (1:2000; Cell Signaling Technology), rabbit anti-STAT3 (1:1000; Cell Signaling Technology); rabbit anti-pSTAT3 (1:1000; Cell Signaling Technology) and rabbit anti-pSMAD1/5/8 (1:1000; Cell Signaling Technology).

Lysosomal pH

80,000 BMDMs were seeded in μ -Slide 8-well high chamber slides (Ibidi) and cultured overnight as previously stated. The next day, cells were washed twice with phenol red-free Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) (containing L-Glutamine and 25 mM HEPES). For the lysosomal and pH staining, the Lysosomal Acidic pH Detection Kit was used (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, LysoPrime Green (1:2000) was first added for 30 min and washed twice with IMDM. Subsequently, pHlys Red was added (1:250) for another 30 min and after removing the medium, BAF (1:1000) was incubated for 30 more min in the corresponding wells. After incubation, cells were washed twice with IMDM, and finally DAPI (1:10,000) was used as a membrane-impermeant viability dye. Cells were then visualized in the live-cell imager Olympus IX83 equipped with a 40x objective and images were analyzed with Olympus CellSens software.

RT-PCR

Total RNA was extracted from adherent cells, liver and BM homogenates using TRIzol™ reagent (Thermo Fisher Scientific) according to the manufacturer's

instructions. Reverse transcription was performed with 2 µg of total RNA using random hexamer primers (200 ng/µl; Roche, Basel, Switzerland), dNTPs (10 mM; GE Healthcare Life Sciences), RNasin (20 U; Promega, Madison, WI, USA), and M-MLV reverse transcriptase (200 U; Invitrogen) in first-strand buffer (Invitrogen). Gene expression of hepcidin antimicrobial peptide (*Hamp*), *Tfr1*, *Fpn1* (*Slc40a1*), divalent metal transporter 1 (*Dmt1*), Hypoxia-inducible factor 1 α (*Hif1\alpha*), heme oxygenase 1 (*Hmox1*), ribosomal protein L4 (*Rpl4*), erythropoietin receptor (*Epor*), signal transducer and activator of transcription 5 (*Stat5*), ferritin heavy chain 1 (*Fth1*), ferritin light chain 1 (*Ftl1*), glutathione peroxidase 4 (*Gpx4*), nitric oxide synthase 2 inducible (*Nos2*), interleukin 6 (*Il6*), interleukin 10 (*Il10*), interleukin 12a (*Il12a*), interleukin 23a (*Il23a*), tumor necrosis factor (*Tnf*) were analyzed by quantitative real-time TaqMan PCR (Eurogentec) while SYBR Green (Thermo Fisher Scientific) was used for nuclear receptor coactivator 4 (*Ncoa4*), six-transmembrane epithelial antigen of the prostate 3 (*Steap3*), solute carrier family 39 member 14 (*Slc39a14*, formerly known as *Zip14*), janus kinase 2 (*Jak2*), and Bcl2-like 1 (*Bcl2l1*). QuantStudio™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) was used following the user's manual. Relative expression levels were calculated using the $\Delta\Delta C_t$ method. *Rpl4* and *Gus\beta* were used as the housekeeping reference genes. The following primers were used (primer forward, primer reverse, and probe (when TaqMan was used)):

Hamp: 5'-TGTCTCCTGCTTCTCCTCCTTG-3'; 5'-AGCTCTGTAGTCTGTCTCATCTGTTGA-3'; FAM-5'-CCAACTTCCCCATCTGCATCTTCTGC-3'
Tfr1: 5'-CGCTTTGGGTGCTGGTG-3'; 5'-GGGCAAGTTTCAACAGAAGACC-3'; FAM-5'-CCCACACTGGACTTCGCCGCA-3'
Fpn1: 5'-CTACCATTAGAAGAATTGACCAGCT-3'; 5'-CAAATGTCATAATCTGGCCGA-3'; FAM-5'-CAACATCCTGGCCCCCATGGC-3'
Dmt1: 5'-GGACTGTGGACGCTCGGTAA-3'; 5'-AATGTTGCCACCGCTGGT-3'; FAM-5'-CATCTCGAAAGTCCTGCTGAGCGAAGA-3'
Hif1\alpha: 5'-GGTTGGTTACTGTTGGTATCATAGACAT-3'; 5'-CAGATTGAAGTGCACCCTAACAAG-3'; FAM-5'-TGCAGTGAAGCACCTTCCACGTTGCT-3'
Hif2\alpha: 5'-CCAACAGAGGCCGGACTGT-3'; 5'-GGCAGTTGTTGTAGACTCTCACTTG-3'; FAM-5'-CCACCTGGAAGGTCCTGCACTGCA-3'
Hmox1: 5'-GTGATGGAGCGTCCACAGC-3'; 5'-TGGTGGCCTCCTTCAAGG-3'; FAM-5'-CGACAGCATGCCCCAGGATTTGTC-3'
Ncoa4: 5'-AGAACGTAACCTTTCGCCCC-3'; 5'-CGATTCTGGTAGTCTGTTCTTCTC-3'
Steap3: 5'-AGGCTTCCTGCTGTTCTTCT-3'; 5'-TCTGGTGAGGATGGTGATGT-3'
Zip14: 5'-ATTGCCCTAGCCGATATGTTC-3'; 5'-TGCCCTGAATACATTGTGAGG-3'
Rpl4: 5'-CGCTGGTGGTGGAAGATAAGG-3'; 5'-CGGTTTCTCATTTTGCCCTTG-3'; Cy5-5'-CAGCCTCCTTGGTCTTCTTGTAACCTTC-3'
Epor: 5'-AGTGAGCATGCCCAGGACA-3'; 5'-AAGTCAGACGGGCAGGAAGAT-3'; FAM-5'-TGGATAAGTGGTTGCTGCCCCGG-3'
Jak2: 5'-CCACGGCCCAATATCAATG-3'; 5'-CCCGCCTTCTTTAGTTTGCTA-3'
Bcl2l1: 5'-GCATCTTCTCCTTCCAGC CT-3'; 5'-CACCTACCCAGCCTCCGTTA-3'
Fth1: 5'-GCGAGGTGGCCGAATCT-3'; 5'-CAGCCCGCTCTCCCAGT-3'; FAM-5'-CCTGCAGGATATAAAGAAACCAGACCGTGA-3'
Ftl1: 5'-GAAACTCATCAAGAAGATGGGCA-3'; 5'-TGGTTGTGGCCCCGC-3'; FAM-5'-CCATCTGACCAACCTCCGCAGGGT-3'

Gpx4: 5'-GATGGA GCCCATTCCTGAACC-3'; 5'-CCCTGTACTTATCCAGGCAGA-3';
 FAM-5'-ATTCTCAGCCAAGGACATCGA-3'
Gusβ: 5'-CTCATCTGGAATTTGCCGA-3'; 5'-GGCGAGTGAAGATCCCCTTC-3';
 FAM-5'-CGAACCAGTCACCGCTGAGAGTAATCG-3'
Nos2: 5'-CAGCTGGGCTGTACAAACCTT-3'; 5'-CATTGGAAGTGAAGCGTTTCG-3';
 FAM-5'-CGG GCAGCCTGTGAGACCTTTGA-3'
Il6: 5'-TGTTCTCTGGGAAATCGTGGA-3'; 5'-AAGTGCATCATCGTTGTTTCATACA-3';
 FAM-5'-ATGAGAAAAGAGTTGTGCAATGGCAATTCTG-3'
Il10: 5'-CCAGAGCCACATGCTCCTAGA-3'; 5'-
 TGGTCCTTTGTTTGAAAGAAAGTCT-3'; FAM-5'-TGCGGACTGCCTTCAGCCAGG-
 3'
Il12a: 5'-TACTAGAGAGACTTCTTCCACAACAAGAG-3'; 5'-
 TCTGGTACATCTTCAAGTCCTCATAGA-3'; FAM-5'-
 AGACGTCTTTGATGATGACCCTGTGCCT-3'
Il23a: 5'-AGCGGGACATATGAATCTACTAAGAGA-3'; 5'-
 GTCCTAGTAGGGAGGTGTGAAGTTG-3'; FAM-5'-
 CCAGTTCTGCTTGCAAAGGATCCGC-3'
Tnf: 5'-TTCTATGGCCCAGACCCTCA-3'; 5'-TTGCTACGACGTGGGCTACA-3';
 FAM-5'-CTCAGATCATCTTCTCAAAATTCGAGTGACAAGC-3'

Labile iron pool by Calcein AM

700,000 cells were seeded overnight in 6-well plates as described in the previous sections. The next day, 25 µM FeN was added for 4 and 24 h and after incubation, cells were washed twice with PBS. Subsequently, cells were incubated for 5 min with Calcein AM (1 µg/mL; Thermo Fisher Scientific) and fluorescence intensity was measured using a Spark multimode microplate reader (Tecan, Männedorf, Switzerland) following the manufacturer's instructions. After, 100 µM of deferoxamine (DFO), an iron chelator, was added for 1 h. Samples were measured again in the microplate reader. Labile iron pool (LIP) was then calculated as the difference between Calcein AM fluorescence intensity and after adding DFO.

Atomic absorption spectrometry (AAS)

The cell pellets were washed twice with 1 mL of PBS and stored at -20 °C until analysis. After thawing, the pellets were resuspended in 200 µL of Milli-Q water containing 0.2% Triton X-100 and lysed by sonication using a cup booster (Sonopuls; Bandelin, Berlin, Germany). Sonication was performed three times for 120 s at 65% power, cycle 8, with cooling at 4 °C. The iron content of the cell lysates was determined by graphite furnace-AAS using Extended Lifetime Graphite Cuvettes (Thermo Fisher Scientific). Measurements were carried out at 248.3 nm with a 0.2 nm bandpass under an argon atmosphere, applying Zeeman background correction. Calibration solutions ranging from 0.5 to 12.5 µg/L were prepared by serial dilution of a 1000 mg/L iron standard (TraceCERT; Sigma-Aldrich) in 0.2% ultrapure nitric acid and Milli-Q water.

Intracellular iron quantification using the live-cell fluorescent microscopy

BMDMs were seeded in DMEM containing 10% FCS with 2 mmol/L L-glutamine, 1% P/S overnight. RPMs were mechanically dissociated from the spleen with a cell strainer, treated with Ery Lysis to lyse RBCs, and then incubated in DMEM. Next day, the supernatant was removed and the cells were washed twice with phenol red-free

medium. Cells were left untreated for 16 h, after which cells' supernatant was discarded and the cells washed twice. Lyso-FerroRed (Dojindo), previously diluted in DMSO according to the manufacturer's instructions, was diluted in phenol red-free media (1:1000) containing 2% FCS. FerroOrange (Dojindo), diluted in DMSO according to the manufacturer's instructions, was diluted to a final concentration of 1 μ M.² Finally, LysoTracker (Thermo Fischer Scientific), also diluted in DMSO, and added at a final concentration of 100 nM. All three reagents were incubated for 30 min at 37 °C and 5% CO₂. After incubation, cells were washed twice and observed under the live-cell imager Olympus IX83 equipped with a 40x objective. Viability was measured with Calcein-AM (esterase activity). Single-cell fluorescence quantification was performed using the scanR analysis software (Evident). Individual cells were segmented based on the cellular mask, and fluorescent subcellular structures were identified using marker-specific segmentation parameters. For each cell, the integrated fluorescence intensity of all detected subcellular objects was quantified and normalized to their cumulative object area, yielding the mean fluorescence intensity within the respective subcellular compartment. Identical analysis parameters were applied to all images within an experiment. For spleen tissue, the analysis was restricted to red pulp macrophages, which were identified by gating on DAPI-positive, F4/80-positive cells. For each experimental group, several hundred cells were analyzed across three technical replicate wells, with four imaging fields acquired per well. For statistical analysis, the mean value of all analyzed cells within each well was calculated, and each dot in the corresponding plots represents the mean of one technical replicate well. Images were analyzed with Olympus CellSens software.

Transferrin uptake

By flow cytometry: BMDMs were seeded overnight in 6-well plates as previously described in section "Production and seeding of bone marrow-derived macrophages". The next day, transferrin (Tf) conjugated-Alexa Fluor™ 488 (Tf-Af488; Thermo Fisher Scientific) was diluted to 25 μ g/mL in DMEM with 1% FCS and added to the cells for 20 min at 37 °C. After incubation, the cells were placed on ice to halt endocytosis and recycling and washed with cold PBS twice. Subsequently, the cells were incubated with 2.5 mM EDTA in PBS for 15 min and harvested by gentle scraping. The cells were then centrifuged at maximum speed for 10 s and resuspended in FACS buffer containing conjugated antibodies F4/80-APC (1:200; BioLegend), CD11b-APC-A750 (1:200; BioLegend) and CD45-PE (1:200; BD Biosciences). Cells were incubated for 15 min at 4 °C in the dark. Following incubation, cells were washed once with 1 mL FACS buffer, centrifuged and resuspended in 100 μ L FACS buffer containing DAPI (1:50,000). Flow cytometry was conducted with a CytoFlex S™ flow cytometer. Cells were gated based on FSC-A vs SSC-A. Singlets were identified by gating FSC-A vs FSC-H. Live cells were selected from the DAPI-negative population. Leukocytes were identified as CD45⁺ cells. From these, macrophages were identified by gating for F4/80⁺CD11b⁺ cells. Finally, the median fluorescence intensity (MFI) of Tf-AF488 was measured in the FITC channel within the selected macrophage population and analyzed with FlowJo 10.7.0 software.

By immunofluorescence microscopy: 80,000 cells were seeded in Poly-D-Lysine-treated (0.1 mg/mL; Gibco, Thermo Fisher Scientific) high-precision coverslips (12 mm diameter, 1.5H; Marienfeld, Lauda-Königshofen, Germany). Next day, 25 μ g/mL of Tf-AF488 was added for 20 min at 37 °C and afterwards washed with PBS twice. Cells were then fixed with 4% paraformaldehyde (PFA; containing 4% D-saccharose [Sigma-Aldrich] in 0.2 M sodium phosphate buffer [pH 7.2]) for 10 min and washed three times

with PBS. The day after, coverslips were placed on wet chambers and were permeabilized for 30 min with 0.2% Triton X-100. After permeabilization, AF-594-phalloidin (1:400, Thermo Fisher Scientific) was added for 1 h at RT and subsequently washed three times with PBS. Slides were then treated with TrueBlack® (Biotium; Fremont, CA, USA) according to the manufacturer's instructions and afterwards washed three more times with PBS. Finally, slides were mounted with mounting media-containing DAPI, observed in Olympus BX61VS slide scanner equipped with a 40x/0.75 NA objective and analyzed with Olympus CellSens software.

Tf-AF488 pulse-chase

BMDMs were seeded onto 1.5H high-precision coverslips coated with Poly-D-Lysine, on three 24-well plates with medium containing 1% FCS and incubated overnight. The next day, the plates were washed with cold PBS and placed on ice to cool down. When cold, 25 µg/mL of conjugated Tf-AF488 were added for 25 minutes. For the 0-min time point plate, cells were washed twice with cold PBS, fixed with 4% PFA for 10 min at RT, and washed three times for 5 min. The remaining two plates were put in the incubator at 37 °C for 5 and 15 min to follow Tf-AF488 endocytic traffic. After incubation, cells were washed with cold PBS, fixed with 4% PFA, and washed again. The following day, coverslips were transferred to wet chambers for staining. First, cells were incubated with blocking buffer for 1 h at RT and washed. After, the primary antibodies rat anti-mouse LAMP1 (1:200; BD Pharmingen), and rabbit anti-mouse EEA1 (1:200; Santa Cruz Biotechnology, Dallas, TX, USA) were added for 1 h at RT. After washing three times for 5 min, the secondary antibodies goat anti-rabbit AF568 (1:1000; Invitrogen), and goat anti-rat AF568 (1:1000; Invitrogen) were added for another hour at RT. After washing three times for 5 min, cells were stained with Hoechst (1:10,000; Invitrogen) for 1 min and washed again five times for 5 min. Finally, slides were mounted with Mowiol (Sigma-Aldrich) and imaged using a Leica SP8 confocal microscope (Leica Microsystems; Wetzlar, Germany) equipped with 93x glycerol immersion objective. Images were processed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

80,000 BMDMs were seeded onto 0.1 mg/mL Poly-D-Lysine-treated high-precision coverslips (12 mm diameter, 1.5H). Next day, BAF (10 nM) and FeN (25 µM) were added for 12 h. After the incubation, cells were fixed in 4% PFA for 20 min and washed with PBS. The following day, coverslips were placed in light-protected wet chambers and cells were stained. For that, cells were first incubated with 5% horse serum in washing buffer (0.1% BSA IgG free and 0.2% Triton X-100 [Sigma-Aldrich] in PBS) for 1 h to reduce non-specific binding. After, cells were washed three times with washing buffer and rat anti-mouse LAMP1 (1:200; BD Pharmingen, San Diego, CA, USA) was added overnight at 4 °C. Next day, cells were washed three times and secondary antibody goat anti-rat AF488 (1:1000; Thermo Fisher Scientific) was added for 1 h at RT. After the incubation, cells were washed three times and rat anti-mouse AF647-CD71 conjugated antibody (1:100; BD Pharmingen) was added overnight at 4 °C. Next day, cells were washed three times and AF594-Phalloidin (1:400; Thermo Fisher Scientific) was added for 1 h at RT. After washing three times, coverslips were mounted with mounting medium containing DAPI. Images were taken using the Leica SP8 confocal microscope equipped with 93x glycerol-immersion objective. Images were

processed using Huygens (Scientific Volume Imaging, Hilversum, Netherlands) and ImageJ.

Spleen immunofluorescence:

Paraffin-embedded spleen sections were deparaffinized in xylene (2 × 5 min), followed by rehydration through a graded ethanol series (96% ethanol, 2 × 5 min; 80% ethanol, 5 min) and rinsing in running tap water for 5 min. Antigen retrieval was performed in 1× citrate buffer. Briefly, citrate buffer was heated in a microwave until just before boiling. Slides were then placed in the heated buffer and microwaved intermittently for 15 min, stopping before visible bubbling occurred. Slides were subsequently left to cool in citrate buffer for 20 min at room temperature. Sections were placed in a humidified chamber, rinsed with PBS, and marked around the tissue using a hydrophobic pen, avoiding tissue drying. Sections were then permeabilized and blocked for 1 h at RT in blocking buffer consisting of 5% horse serum, 0.2% BSA, and 0.2% Triton X-100 in PBS. After washing with washing buffer (0.2% BSA and 0.2% Triton X-100 in PBS), sections were incubated with rabbit anti-F4/80 (1:200; Cell Signaling Technology) overnight at 4 °C. After washing, sections were incubated with a secondary antibody goat anti-rabbit AF647 (1:1,000; Thermo Fisher Scientific) for 1 h at RT. Following final washes, slides were mounted using fluorescence mounting medium containing DAPI and sealed with coverslips. Images were obtained using the Olympus BX61VS slide scanner equipped with a 20x/0.75 NA objective.

RBC integrity by flow cytometry

Blood was collected from LMC and LAMTOR2 KO mice into tubes containing 1.5 µL of heparin. For phosphatidylserine (PS) detection, whole blood was diluted in 50 µL of Annexin V binding buffer (Invitrogen) and incubated with Annexin V-APC (1:200; Invitrogen) for 15 min at RT in the dark. Samples were washed and then incubated with Ter119-FITC (1:200; eBioscience) and CD47-PE (1:200; BD Biosciences). Cells were then resuspended in buffer and analyzed by flow cytometry. For analysis, debris was excluded based on FSC-A vs SSC-A. Singlets were identified by gating FSC-A vs FSC-H. RBCs were identified as Ter119⁺ cells. PS exposure was quantified as the percentage of Annexin V⁺ in Ter119⁺ cells and CD47 as well.

Erythrophagocytosis in BMDMs

Erythrophagocytosis was assessed in BMDMs. Briefly, 500,000 BMDMs were seeded per dish. RBCs were isolated from LMC and LAMTOR2 KO mice, diluted in Diluent C (Sigma-Aldrich), and labeled with CellVue Claret (Sigma-Aldrich) according to the manufacturer's instructions. Labeled RBCs were added to BMDMs at a ratio of 2.5 million erythrocytes per 500,000 BMDMs and incubated for 18 h. Cells were then harvested and stained with F4/80-Pacific Blue and 7-AAD to exclude dead cells. Samples were acquired by flow cytometry, and erythrophagocytosis was quantified as the percentage of Claret-positive F4/80⁺ viable macrophages. Samples were acquired on a CytoFLEX™ flow cytometer and analyzed using FlowJo v10.7.0.

RPMs flow cytometry

Spleens were harvested and mechanically dissociated by mashing the tissue using a 100- μ m cell strainer in PBS. Erythrocyte lysis was done with ACK buffer (150 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA , all from Sigma-Aldrich). Single-cell suspension was centrifuged, washed, and stained with CD45-APC (1:400; eBioscience), Gr-1-PerCP (1:200; BioLegend), Ly6C-Pacific Orange (1:800), F4/80-PE-Cy7 (1:200; BioLegend), CD11b-APC-Cy7 (1:200; BioLegend), TIM4-PE (1:200; BioLegend) or CD172a-PE (1:200; BioLegend), and CD71-Pacific Blue (1:100). Samples were acquired on a CytoFLEX™ flow cytometer and analyzed using FlowJo v10.7.0. For analysis, debris was excluded based on FSC-A vs SSC-A. Singlets were identified by gating FSC-A vs FSC-H. RPMs were identified as CD45^+ , after exclusion of Gr-1^+ and Ly6C^+ , and from these population, as $\text{F4/80}^+\text{CD11b}^-$ cells. The percentages of TIM4^+ and CD172a^+ cells were determined within the RPM population, whereas CD71 expression was quantified as MFI.

BM macrophage count

BM cells were isolated from mouse femurs by centrifugation at maximum speed for 10 s. The resulting cell pellet was resuspended in PBS and passed through a 40- μ m cell strainer to obtain a single-cell suspension. For exclusion of lineage-positive cells, BM cells were stained with biotin-conjugated antibodies against CD19, CD3, and CD49b (all 1:200; BioLegend). Biotin-labelled antibodies were detected using Streptavidin-PE-eFluor 610 (1:200; eBioscience). Cells were additionally stained with antibodies against CD169-APC, F4/80-PE-Cy7, CD11b-APC-Cy7, and CD64-PE (all 1:200; BioLegend). BM macrophages were identified by flow cytometry after exclusion of lineage-positive cells and were defined as percentage of $\text{CD11b}^-\text{F4/80}^+\text{CD64}^+$ cells of Lin^- . CD169 expression was assessed within the macrophage population as an additional marker of BM-resident macrophages. Samples were acquired on a CytoFLEX™ flow cytometer and analyzed using FlowJo v10.7.0.

Bead phagocytosis

BMDMs were seeded overnight in DMEM with 1% FCS, 1% P/S and 2 mmol/L L-glutamine. The next day, cells were incubated with fluorescent beads (Fluoresbrite YG Microsphere 0.50 μ m, *MOI* 100) for 1 h at 37 °C. After, cells were washed with PBS three times, and harvested by gently scraping. The cell suspension was centrifuged at maximum speed for 10 s, and the pellet resuspended in FACS buffer containing CD11b-PerCp-Cy5 (1:200; BioLegend), F4/80-APC (1:200; BioLegend), and DAPI (1:50,000). Samples were acquired on a CytoFLEX™ flow cytometer and analyzed using FlowJo v10.7.0. For analysis, debris was excluded based on FSC-A vs SSC-A, singlets were identified by gating FSC-A vs FSC-H, live cells were selected as DAPI-negative population, and macrophages were identified as $\text{CD11b}^+\text{F4/80}^+$ cells. From this population, phagocytic cells were identified as YG bead-positive cells. Phagocytosis was quantified as percentage of YG^+ cells among macrophages.

References

1. Teis D, Taub N, Kurzbauer R, et al. p14–MP1-MEK1 signaling regulates endosomal traffic and cellular proliferation during tissue homeostasis. *The Journal of cell biology*. 2006;175(6):861–868.

2. Grubwieser P, Brigo N, Seifert M, et al. Quantification of Macrophage Cellular Ferrous Iron (Fe 2+) Content using a Highly Specific Fluorescent Probe in a Plate-Reader. Bio-protocol. 2024;14(3):

Supplementary figures

Figure S1

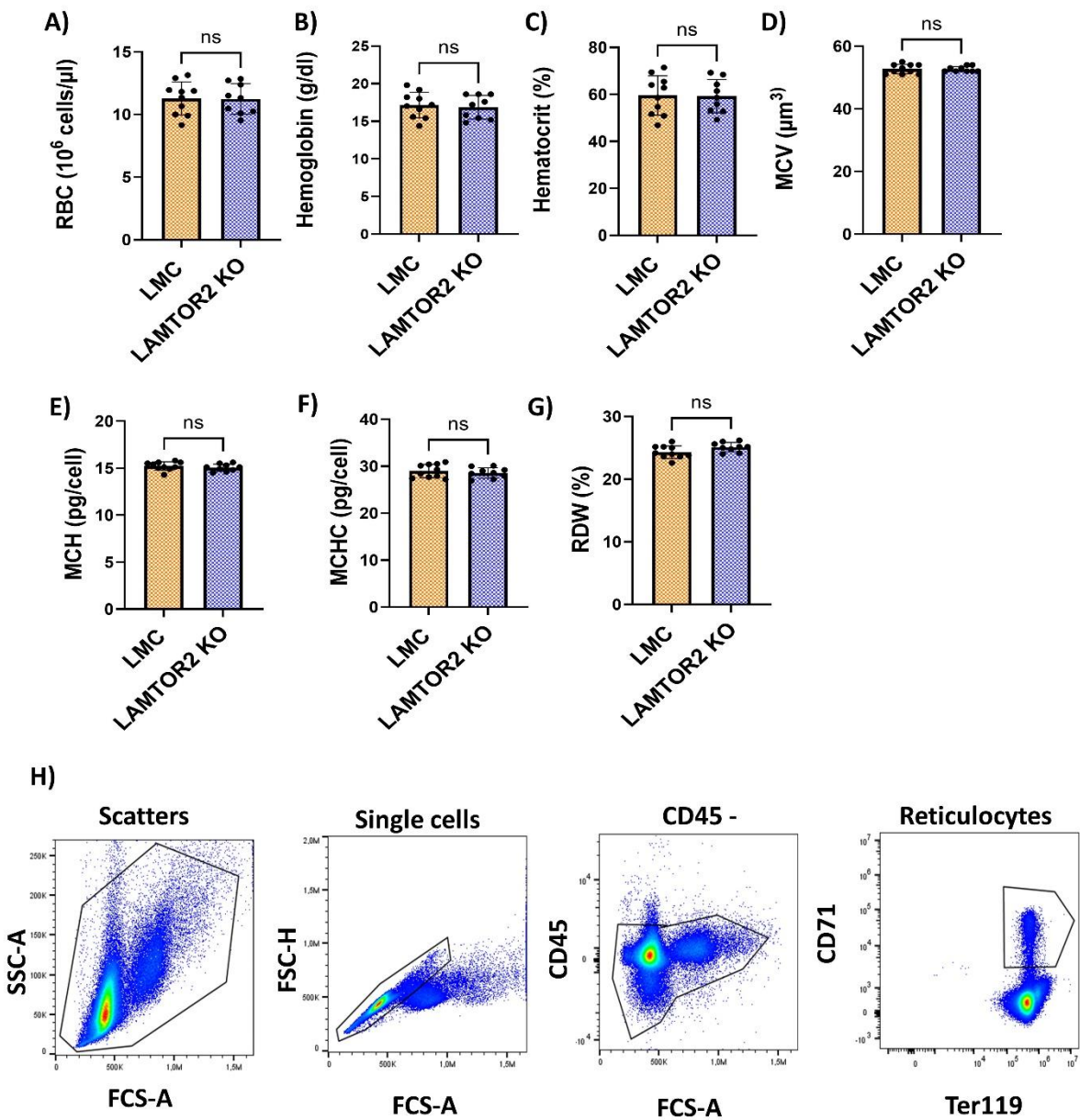


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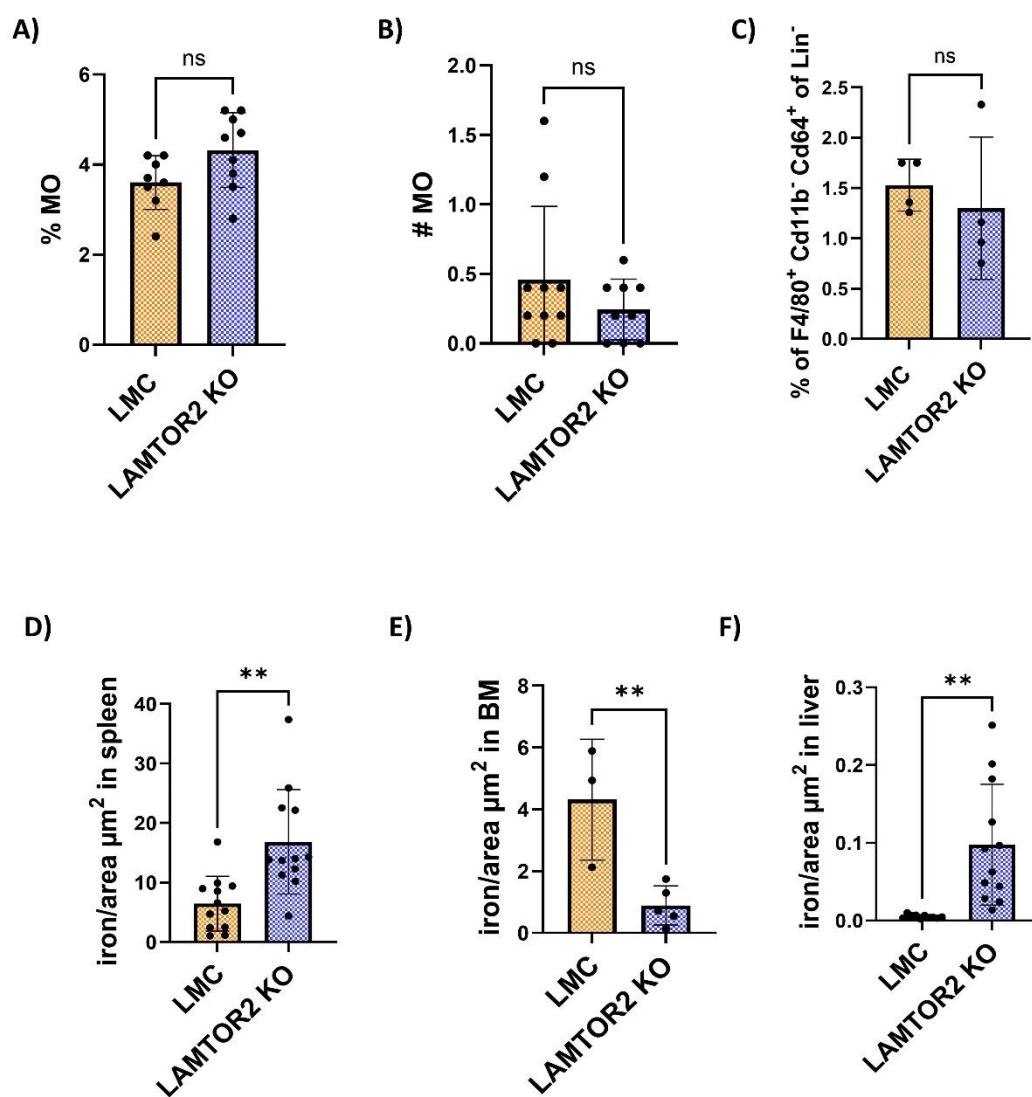


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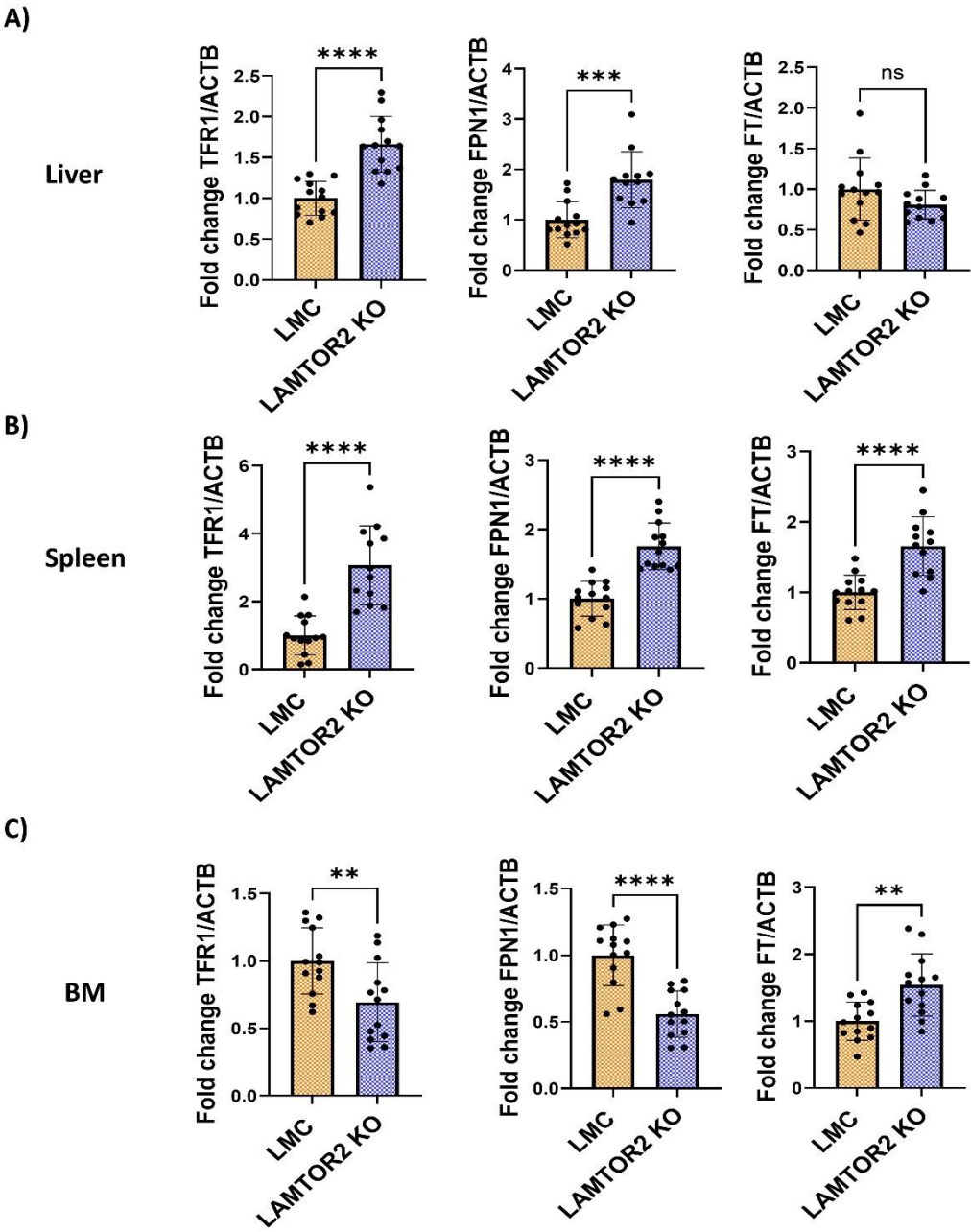


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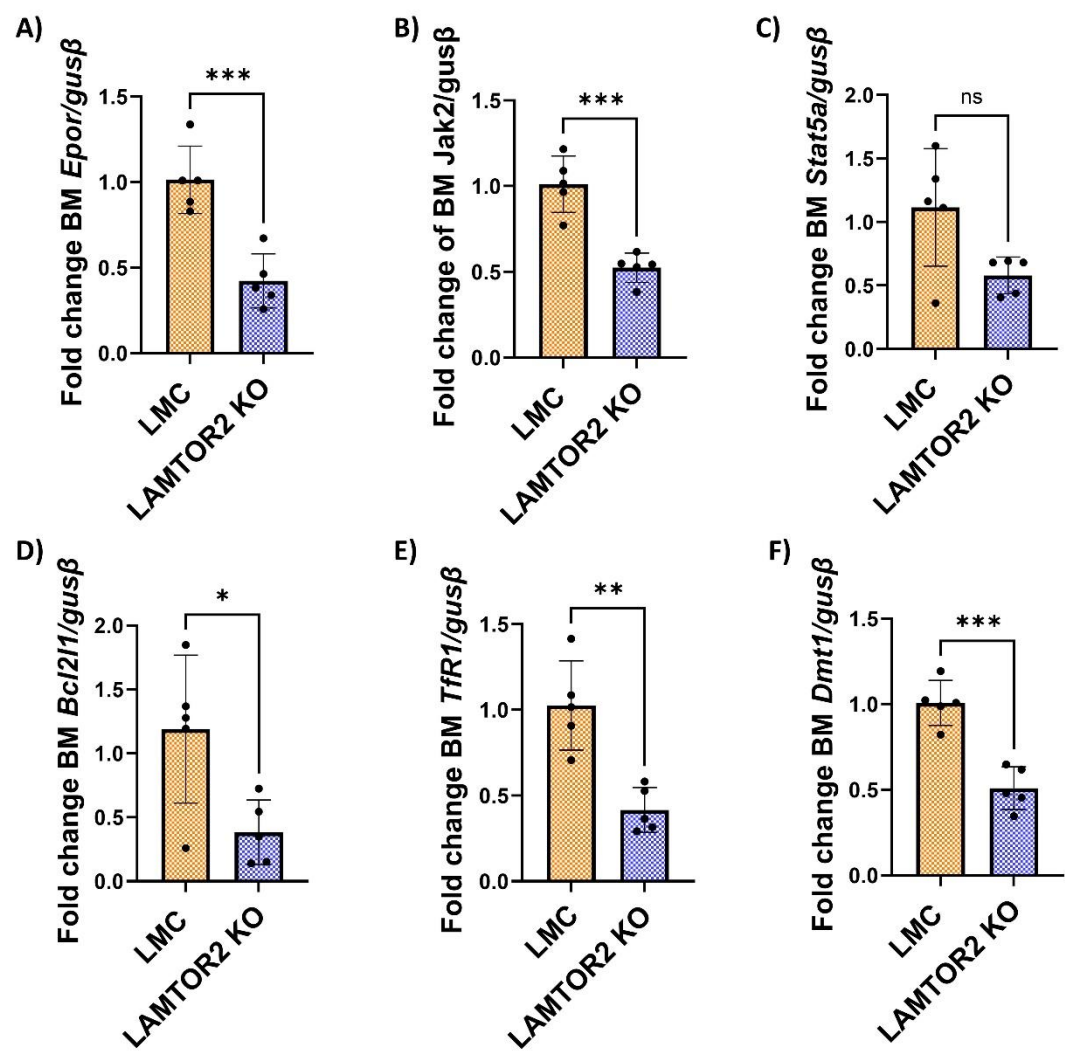


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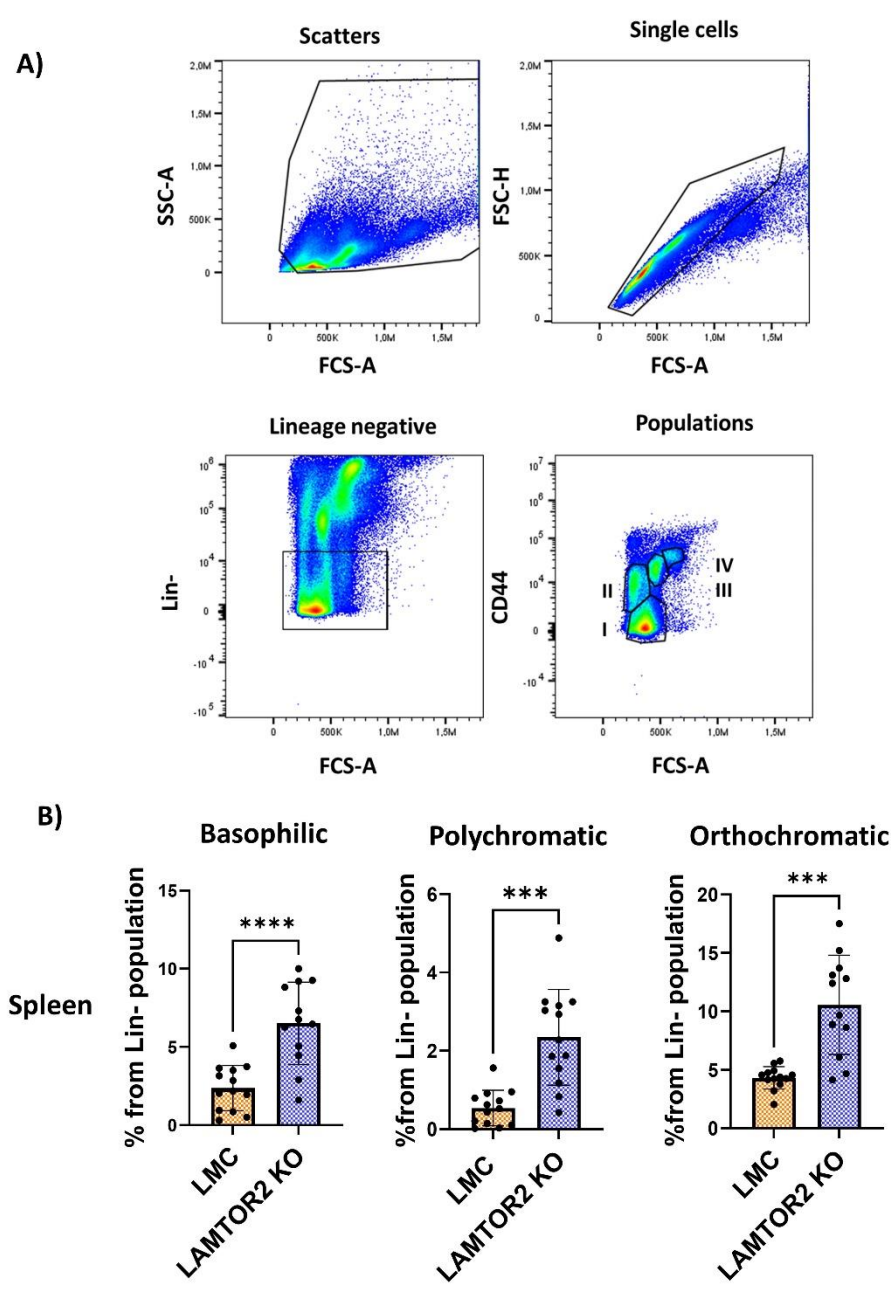


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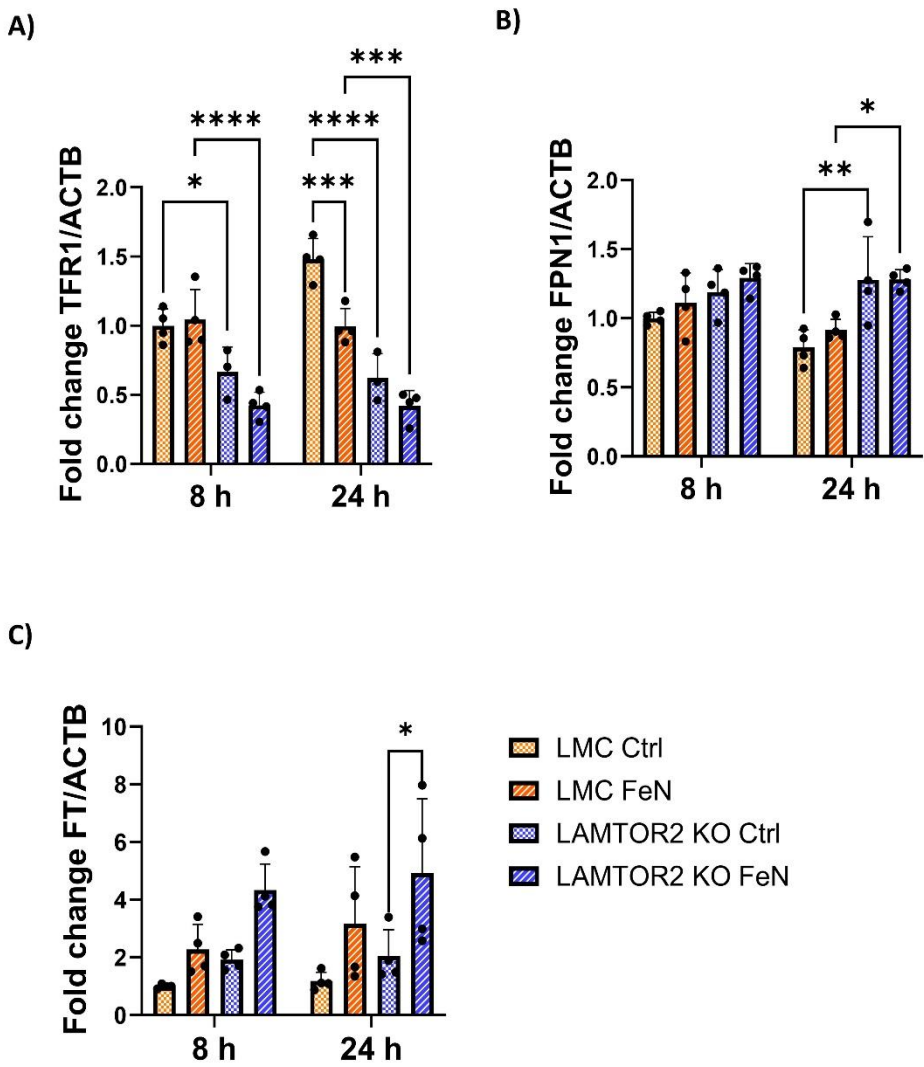


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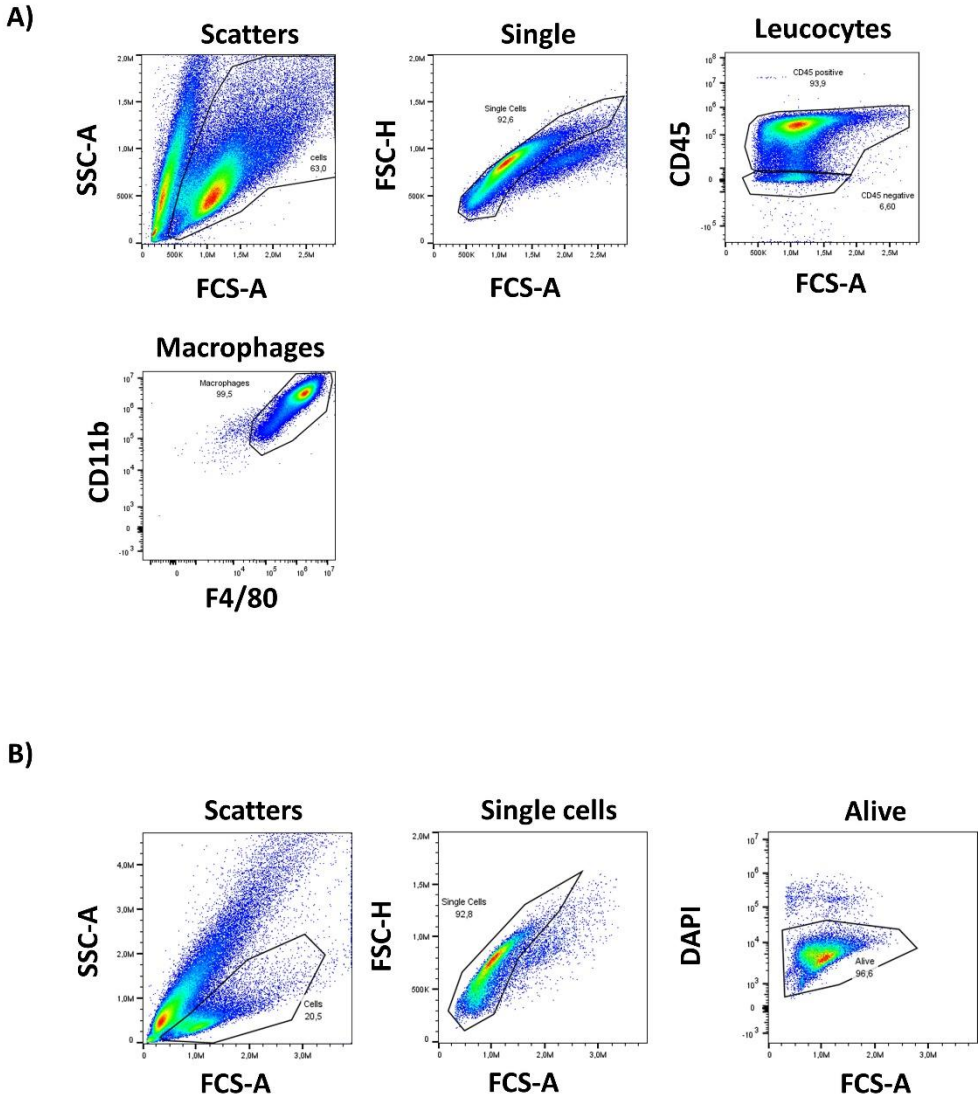


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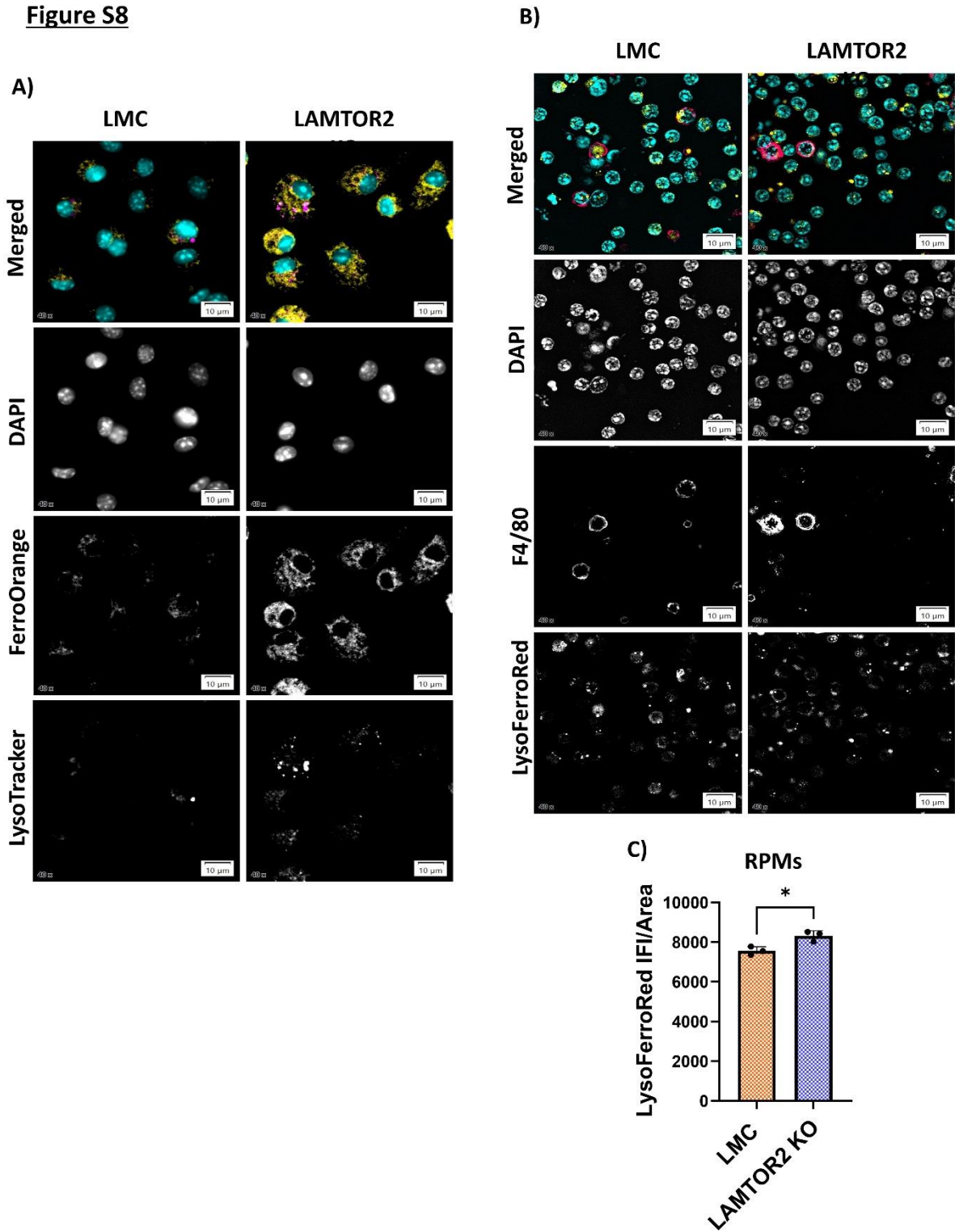


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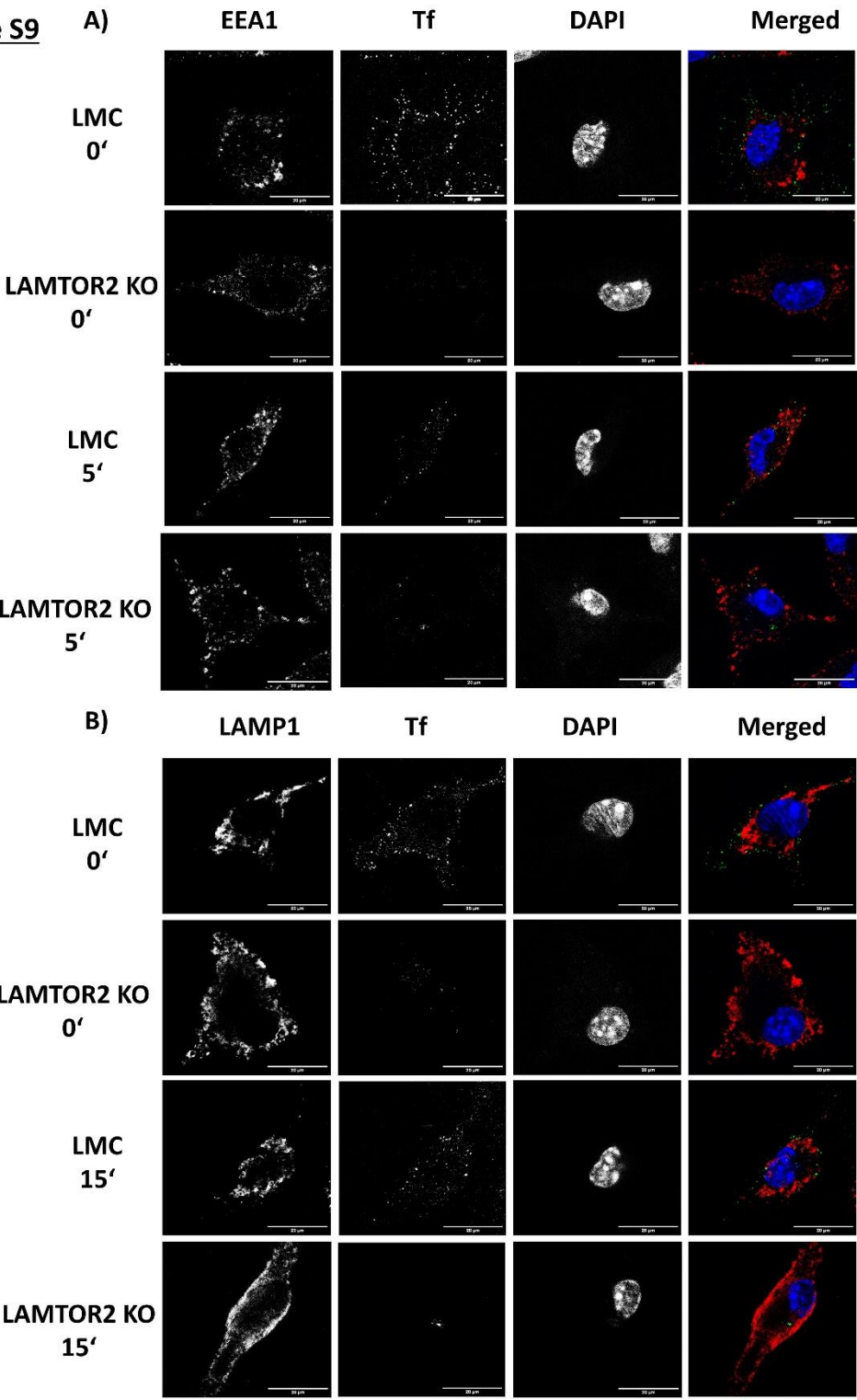


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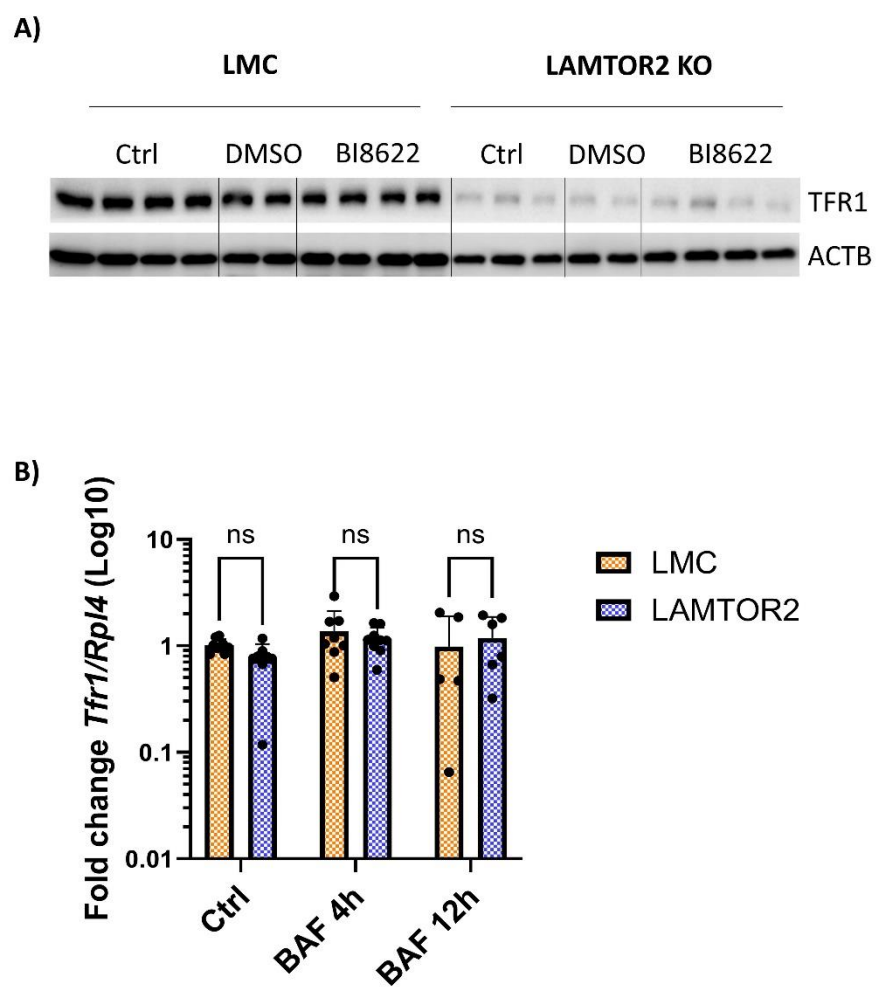


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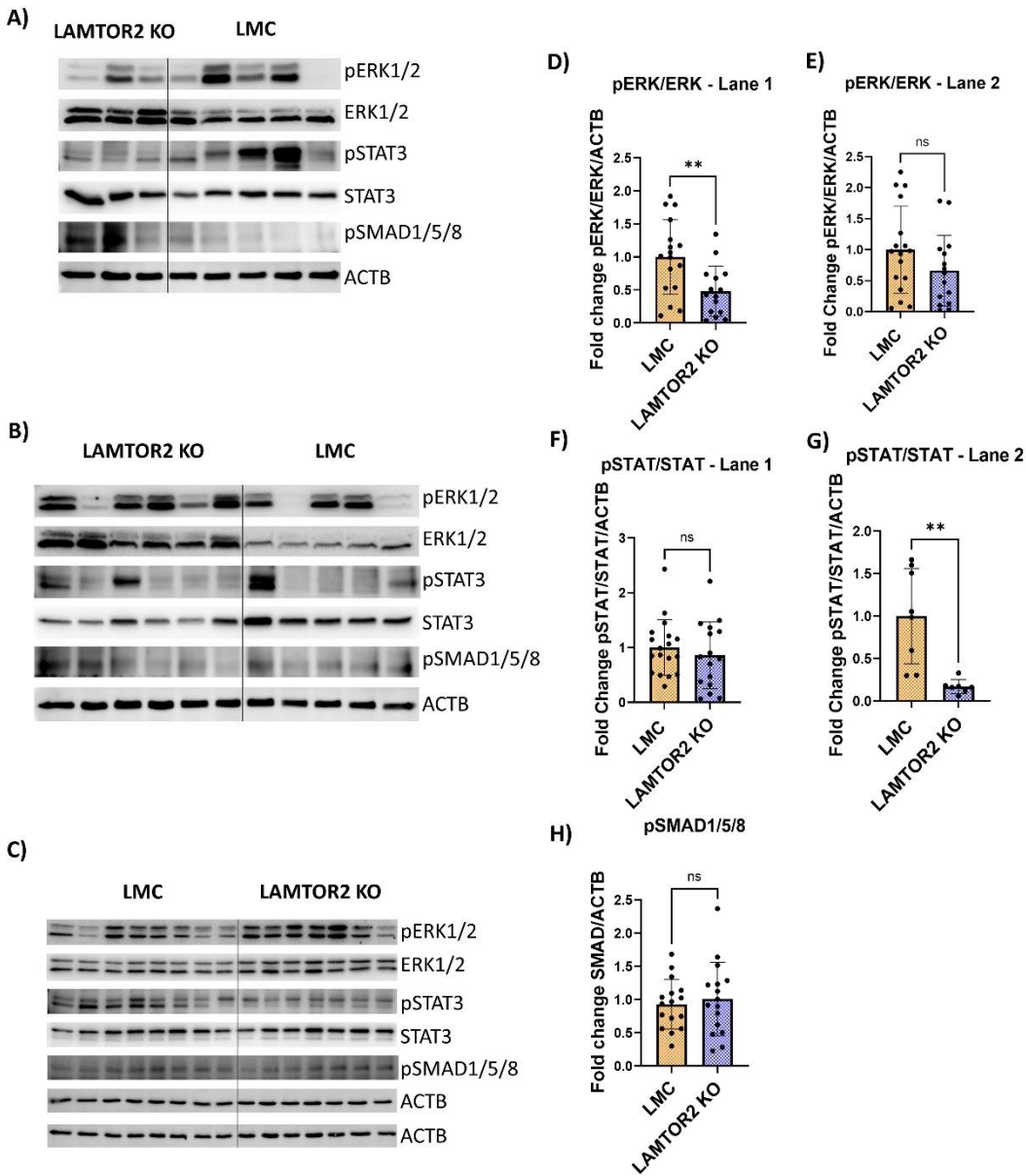


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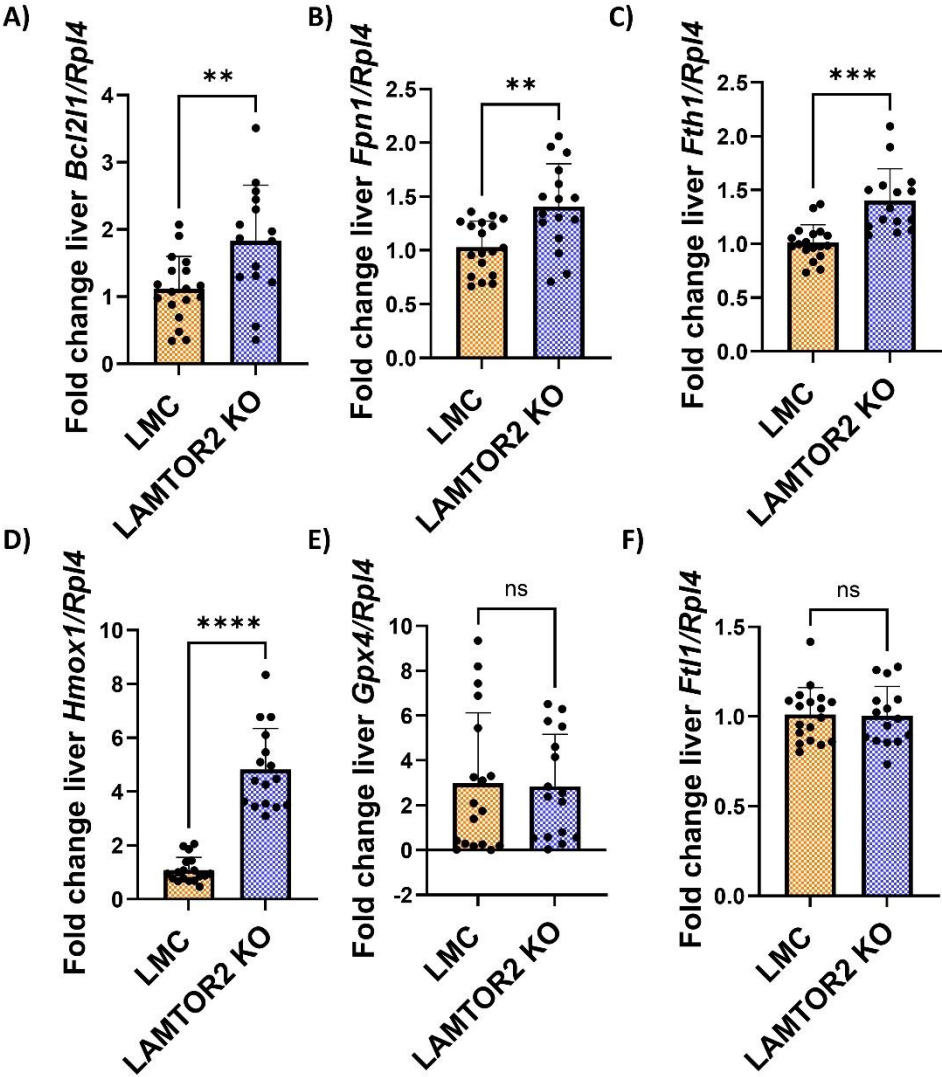


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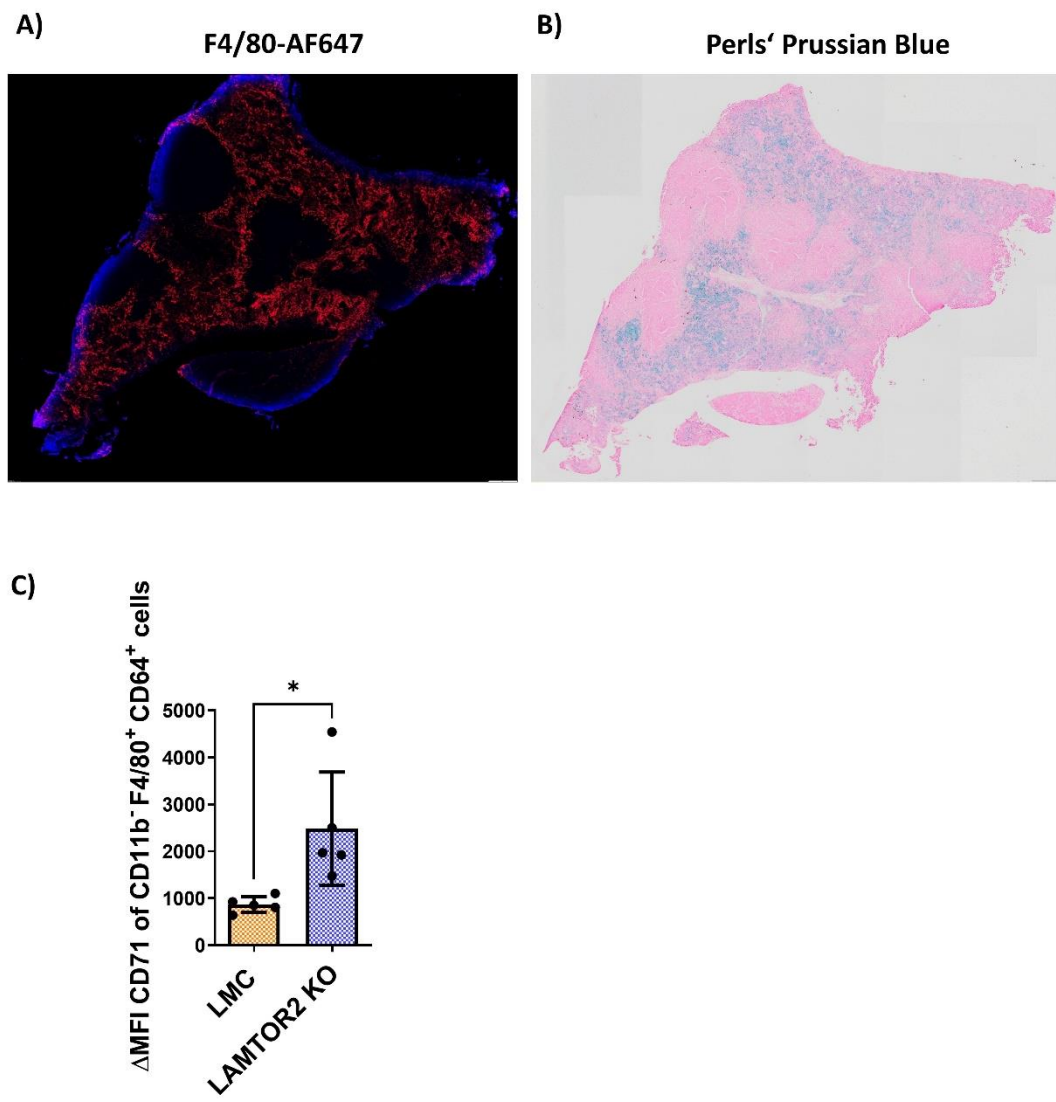


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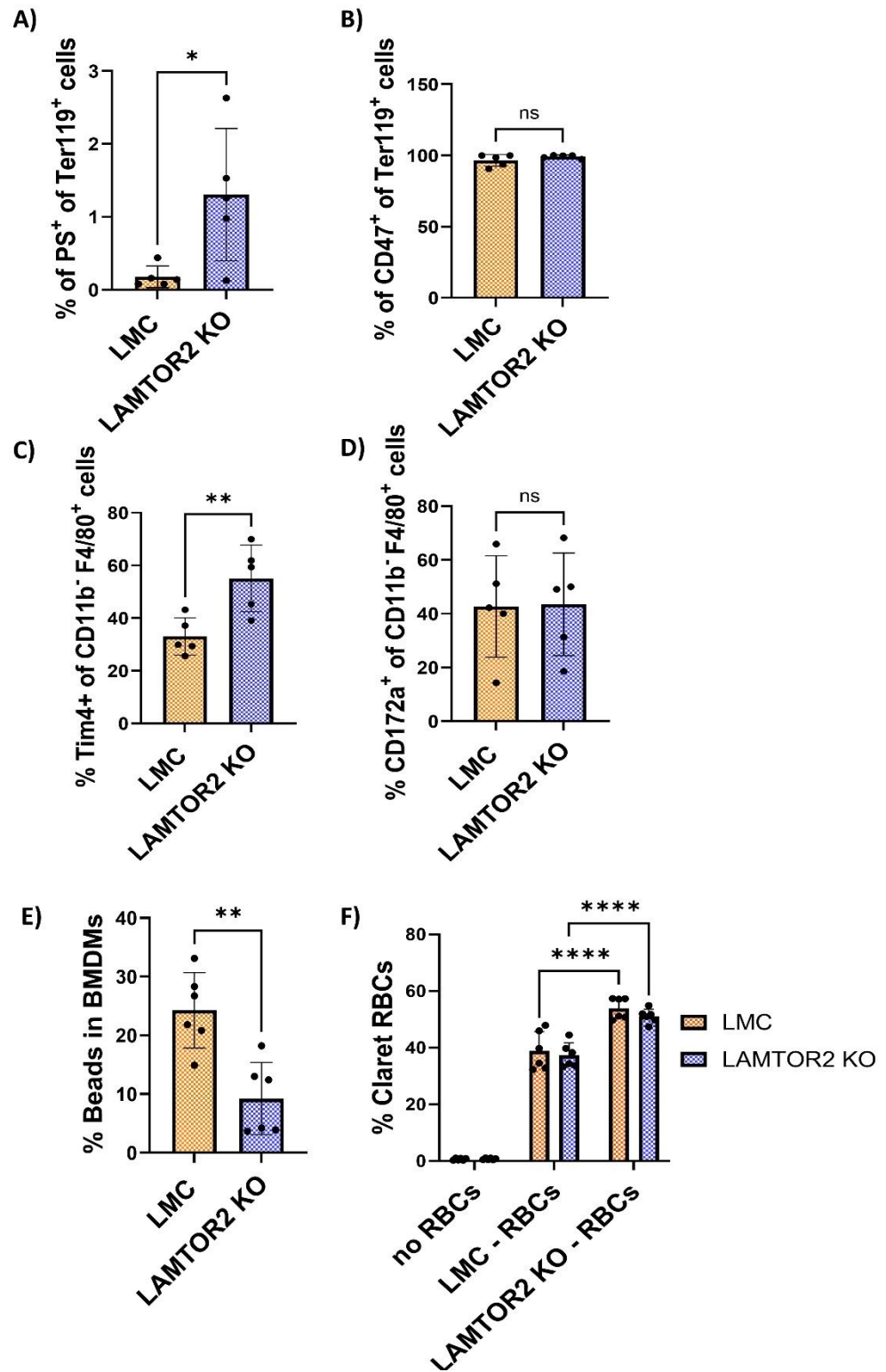
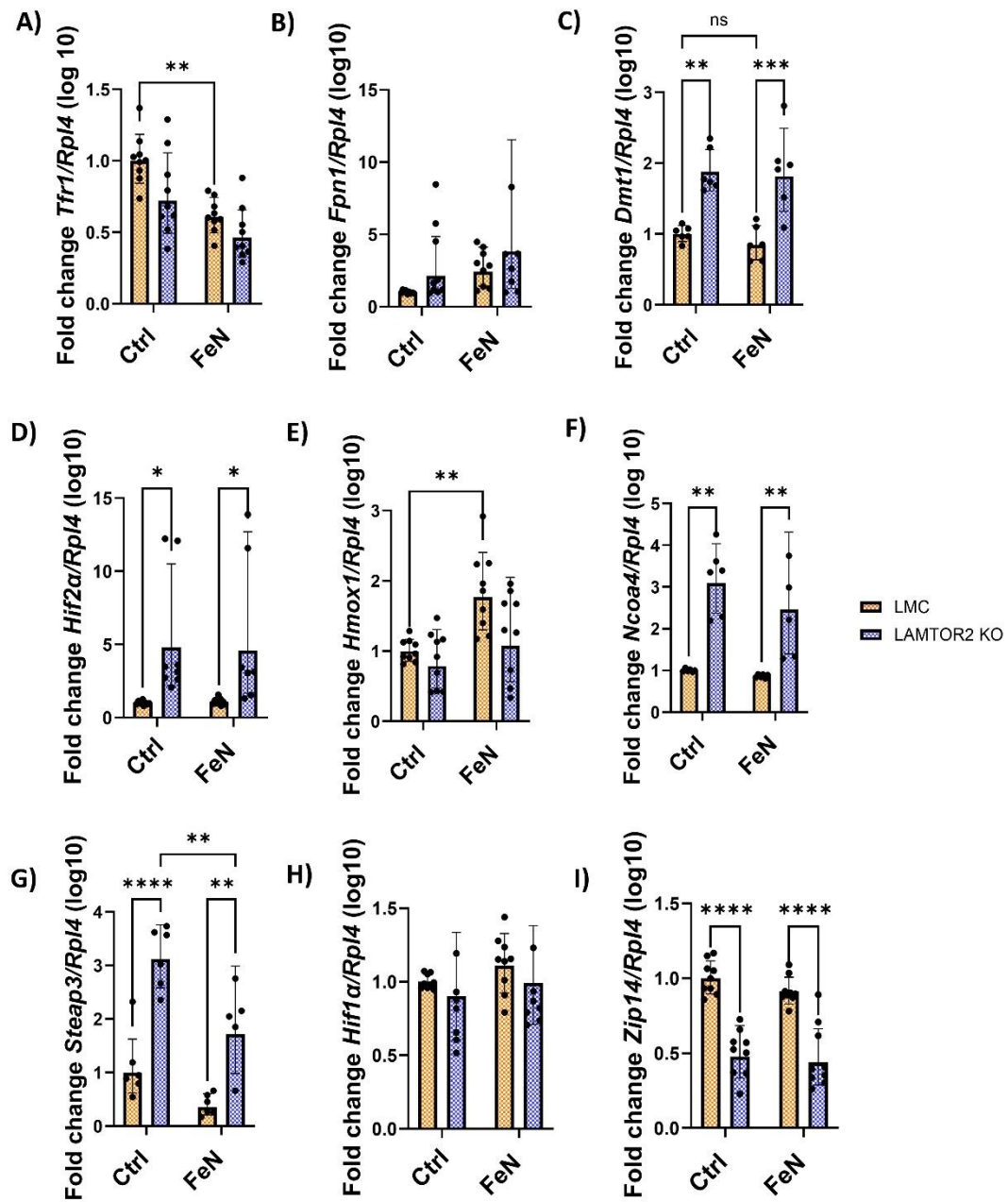


Figure S15



Legends to supplementary figures

Figure S1. Blood count analysis. A) Red blood cell count (RBC). B) Hemoglobin levels. C) Hematocrit percentage. D) Mean corpuscular volume (MCV). E) Mean corpuscular hemoglobin (MCH). F) Mean corpuscular hemoglobin concentration (MCHC). G) Red cell distribution width (RDW). H) Flow cytometry was used to analyze the peripheral blood reticulocytes. The illustrated gating strategy includes sequential gating of forward and side scatter (FSC/SSC), single cells, CD45⁻ cells, and reticulocytes. More details are described in the methods section. Unpaired T-test was applied for panels A to G. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Results are shown as mean ± standard deviation. 10 LysMCre and 9 LAMTOR2 KO mice were analyzed. Results are from two independent experiments.

Figure S2. Macrophage and monocyte (MO) quantification and colorimetric quantification of Prussian Blue staining. A) Percentage of peripheral blood monocytes and B) number of monocytes were quantified by scil VetABC blood cell counter. C) Bone marrow macrophages were quantified as the percentage of lineage negative (Lin⁻) (CD19, CD3, and CD49b), CD11b⁻, F4/80⁺ and CD64⁺ cells by flow cytometric analysis. D) Quantification of staining in spleen. E) Quantification of staining in BM. F) Quantification of staining in liver. More details are described in the methods section. Welch's t-test was employed in panels A, B, D and F. Unpaired T-test was applied in panels C and E. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Results are shown as mean ± standard deviation. 3 to 10 LysMCre (LMC) and 4 to 13 LAMTOR2 KO mice were analyzed. Results are from two independent experiments.

Figure S3. A) Densitometric quantification of liver proteins, where transferrin receptor 1 (TFR1), ferroportin 1 (FPN1), and ferritin (FT) were analyzed. β -Actin (ACTB) was used as a housekeeping protein. B) Densitometric quantification of splenic proteins, where TFR1, FPN1 and FT were analyzed and ACTB was used as a housekeeping protein. C) Densitometric quantification of bone marrow (BM) proteins, where TFR1, FPN1, and FT were analyzed, and ACTB was used as the housekeeping protein. Welch's test was used for panels A-TFR1, A-FT, B-TFR1, C-TFR1. Unpaired T-test was used for panels A-FPN1, B-FPN1, B-FT, C-FPN1, C-FT. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Results are shown as mean ± standard deviation. 13 LysMCre (LMC) and 13 LAMTOR2 KO mice were analyzed. Results are from two independent experiments.

Figure S4. Real time (RT)-PCR of bone marrow (BM). A) Erythropoietin receptor (*Epor*); B) Janus kinase 2 (*Jak2*); C) Signal transducer and activator of transcription 5 (*Stat5*); D) Bcl2-like 1 (*Bcl2l1*); E) Transferrin receptor 1 (*Tfr1*); F) Divalent metal transporter 1 (*Dmt1*). *Gus β* was used as the housekeeping gene to normalize gene expression. Unpaired T-test was applied in panels A, B, E, and F. Welch's t-test was employed in panels C and D. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. 4 LysMCre (LMC) and 5 LAMTOR2 KO mice were analyzed. Results are shown as mean ± standard deviation and are from two independent experiments.

Figure S5. A) Gating strategy employed to identify the erythroid progenitors. More information can be found in the Supplementary methods. In the last panel, the following numbering represents I) red blood cells, II) orthochromatic, III) polychromatic and IV) basophilic. B) Flow cytometry analysis of splenic erythroid progenitors, including basophilic, polychromatic and orthochromatic erythroblasts. Unpaired T-test was applied

in panel B-Polychromatic. Welch's test was applied in panels B-Basophilic and B-Orthochromatic. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. 13 LysMCre (LMC) and 13 LAMTOR2 KO mice were analyzed. Results are shown as mean $\pm$ standard deviation and are from two independent experiments.

Figure S6. Western blot (WB) of bone marrow-derived macrophages (BMDMs) after treatment with 25 μ M FeN for 8 and 24 h. A) Densitometric quantification of transferrin receptor 1 (TFR1). B) Densitometric quantification of ferroportin 1 (FPN1). C) Densitometric quantification of ferritin (FT). β -Actin (ACTB) was used as the housekeeping protein. 2-way ANOVA with Tukey's multiple comparisons test was applied in all panels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation. Data are from two independent experiments performed in duplicates. Each dot represents one technical replicate. LMC = LysMCre.

Figure S7. Flow cytometry was used to gate bone marrow-derived macrophages (BMDMs) used in the transferrin (Tf) assay. A) Sequential gating of FSC/SSC, single cells, CD45⁺ cells and macrophages, identified as CD11b⁺F4/80⁺ cells. B) Simplified gating strategy for bone marrow-derived macrophages (BMDMs), including FSC/SSC, single cells and live cells.

Figure S8. Intracellular labile Fe²⁺ visualized using live-cell fluorescence microscopy. A) Representative images of FerroOrange-stained bone marrow-derived macrophages (BMDMs) isolated from LysMCre (LMC) and LAMTOR2 KO mice. LysoTracker was also used to visualize lysosomes. FerroOrange is represented in yellow, DAPI in blue and LysoTracker in purple. B) Representative images of LysoFerroRed-stained isolated red pulp macrophages (RPMs), which were identified as F4/80⁺ cells. LysoFerroRed is represented in yellow, F4/80 is represented in red and DAPI in blue. Scale bar is 10 μ m. C) Quantification of LysoFerroRed. Lysosomal labile Fe²⁺ was quantified as the integrated fluorescence intensity of LysoFerroRed-positive objects normalized to their cumulative area (IFI/area). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation. Data in panel C are from one independent experiment performed in triplicates. Each dot represents one technical replicate.

Figure S9. Pulse-chase experiment of transferrin (Tf)-AF488 in bone marrow-derived macrophages (BMDMs). A) Cells were incubated with Tf-AF488 for 0 and 5 min prior to fixation. Early endosomes were identified by early endosome antigen 1 (EEA1)-AF594 immunostaining and nuclei were counterstained with DAPI. Representative images are shown. Scale bar 20 μ m. B) Cells were incubated with Tf-AF488 for 0 and 15 min prior to fixation. Lysosomes were labelled with lysosome-associated membrane protein 1 (LAMP1)-AF594 immunostaining and nuclei were counterstained with DAPI.

Figure S10. A) Western blot (WB) of BI8622 (HUWE E3 ubiquitin ligase inhibitor)-treated bone marrow-derived macrophages (BMDMs). LysMCre (LMC) and LAMTOR2 KO BMDMs were incubated with 5 μ M BI8622 for 24 h. BI8622 was diluted in DMSO. Vehicle controls received the corresponding DMSO volume (0.1 μ L/mL). Transferrin receptor 1 (TFR1) and the housekeeping gene β -actin (ACTB) were analyzed. B) Real time (RT)-PCR of *Tfr1* in bafilomycin (BAF)-treated BMDMs. LMC and LAMTOR2 KO BMDMs were incubated with 10 nM BAF for 4 and 24 h. Gene expression of *Tfr1* and the housekeeping gene *Rpl4* were analyzed. Data are represented as log₁₀. 2-way ANOVA with Tukey's multiple comparisons test was employed for data analysis.

Results are shown as mean $\pm$ standard deviation. Results are from two and three independent experiments performed in triplicates. Each dot represents one technical replicate.

Figure S11. Western blot (WB) of hepcidin signaling proteins in mouse liver. A) Experiment 1, where five LysMCre (LMC) and three LAMTOR2 KO mice were used. B) Experiment 2, where five LMC and six LAMTOR2 KO mice were used. C) Experiment 3 where eight LMC and seven LAMTOR2 KO mice were used. Experiments are shown separately because of variability between independent experiments. D) Densitometric quantification of phosphorylated extracellular signal-regulated kinase 1/2 normalized (p-ERK1/2) to (ERK1/2) and β -actin (ACTB) using the bands from the first lane. E) Densitometric quantification of p-ERK1/2 normalized to ERK1/2 and ACTB using the bands from the second lane. F) Densitometric quantification of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) normalized to STAT3 and ACTB using the bands from the first lane. G) Densitometric quantification of p-STAT3 normalized to STAT3 and ACTB using the bands from the second lane. H) Densitometric quantification of phosphorylated SMA and MAD (mothers against decapentaplegic)-related 1/5/8 (pSMAD1/5/8) was normalized to ACTB. Welch's test was employed for all the panels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation and are from three independent experiments.

Figure S12. Real time (RT)-PCR of NRF2 targets in mouse liver. A) Bcl2-like 1 (*Bcl2l1*); B) Ferroportin 1 (*Fpn1*); C) Ferritin heavy chain 1 (*Fth1*); D) Heme oxygenase 1 (*Hmox1*); E) Glutathione peroxidase 4 (*Gpx4*) and F) Ferritin light chain 1 (*Ftl1*). Ribosomal protein L4 (*Rpl4*) was used as a housekeeping gene. Unpaired T-test was applied in panels B, E, and F, and Welch's test was employed in panels A, C, and D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. 18 LysMCre (LMC) and 16 LAMTOR2 KO mice were analyzed. Results are shown as mean $\pm$ standard deviation and are from three independent experiments.

Figure S13. A) Immunofluorescence microscopy image of mouse LAMTOR2 KO spleen, where F4/80 splenic macrophages were stained with Alexa Fluor (AF)-647 (in red). B) Perls' Prussian Blue staining to detect iron (in blue) in LAMTOR2 KO spleen. C) Mean fluorescence intensity (MFI) of CD71 (Transferrin receptor 1) analyzed by flow cytometry in red pulp macrophages (RPMs), which were identified as CD11b⁺ F4/80⁺ CD64⁺ cells. Welch's test was applied in panel C. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation. 5 LysMCre (LMC) and 5 LAMTOR2 KO mice were analyzed. Scale bar = 200 μ m.

Figure S14. Erythrophagocytosis analysis. A) Percentage of phosphatidylserine (PS) positive erythrocytes, which were identified as Ter119⁺ cells by flow cytometry. B) Percentage of CD47⁺ erythrocytes, which were identified as Ter119⁺ cells by flow cytometry. C) Percentage of TIM4⁺ red pulp macrophages (RPMs) which were identified as CD11b⁺ F4/80⁺ cells by flow cytometry. D) Percentage of CD172a positive RPMs which were identified as CD11b⁺ F4/80⁺ cells by flow cytometry. E) Percentage of fluorescent bead-positive bone marrow-derived macrophages (BMDMs) analyzed by flow cytometry; F) Percentage of CellVue Claret-positive BMDMs following incubation with fluorescently labeled red blood cells (RBCs) isolated from LysMCre (LMC) and LAMTOR2 KO mice. RBCs from both LMC and LAMTOR2 KO mice were

used and the experiment was performed by flow cytometry. More details are described in the methods section. Welch's test was applied in panels A and B. Unpaired T-test was applied in panels C, D, and E, and two-way ANOVA with Tukey's multiple comparisons test was used in panel F. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. For panels A, B, C, and D, 5 LysMCre (LMC) and 5 LAMTOR2 KO mice were analyzed. In panels E and F, two independent experiments were performed in triplicates. Each dot represents one technical replicate. Results are shown as mean $\pm$ standard deviation.

Figure S15. Real time (RT)-PCR of iron-related genes in bone marrow-derived macrophages (BMDMs). LysMCre (LMC) and LAMTOR2 KO BMDMs were incubated with ferric nitrate nonahydrate (FeN) for 8 h and gene expression of different genes was analyzed. Ribosomal protein L4 (*Rpl4*) was used as a housekeeping gene. A) Transferrin receptor 1 (*Tfr1*); B) Ferroportin 1 (*Fpn1*); C) Divalent metal transporter 1 (*Dmt1*). D) Hypoxia-inducible factor 2 α (*Hif2 α*). E) Heme oxygenase (*Hmox*). F) Nuclear receptor coactivator 4 (*Ncoa4*). G) Six-transmembrane epithelial antigen of prostate 3 (*Steap3*). H) Hypoxia-inducible factor 1 α (*Hif1 α*). I) Zip14 (*Slc39a14*). Data are represented as log10. 2-way ANOVA with Tukey's multiple comparison test was employed for data analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Results are shown as mean $\pm$ standard deviation and are from two or three independent experiments performed in triplicates. Each dot represents one technical replicate.