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Myeloid-Specific Estrogen Receptor Alpha is Required for Murine Iron Homeostasis

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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular & Medical Pharmacology

by

Amy Helene Fluitt

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Molecular & Medical Pharmacology

University of California, Los Angeles, 2015

Professor Steven J. Bensinger, Co-Chair

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Reticuloendothelial macrophages of the spleen, liver, blood, and bone marrow are responsible for recycling approximately 25 mg of iron from senescent red blood cells each day to provide iron for erythropoiesis. The female sex hormone, estradiol, is thought to influence iron homeostasis in females, yet there is little mechanistic data linking estrogen signaling to macrophage iron metabolism. To investigate this relationship, we crossed floxed *Esr1* and LysM-Cre transgenic animals to excise estrogen receptor alpha/ERα in the myeloid lineage. Female myeloid-specific ERα knockout/MACER mice have reduced red blood cell counts, hemoglobin, and serum iron levels compared to control females. We observed a concomitant increase in the splenic and hepatic iron content of MACER mice and confirmed the presence of iron deposits in the red pulp zone of the spleen. MACER spleens have normal red pulp macrophage staining, which implies that these mice are capable of clearing senescent red blood

cells. However, there are significant alterations in iron metabolism gene expression in MACER splenocytes that are consistent with iron accumulation. Macrophages express high levels of ferroportin and ferritin under iron overload conditions to facilitate the export and storage of excess iron, respectively. We also observed elevated hemoxygenase 1 expression in MACER spleens, which catabolizes free heme to avoid heme-related toxicity. The gene expression profiles suggest that free iron and heme accumulate in MACER red pulp macrophages following the phagocytosis of senescent red blood cells and hemoglobin breakdown. Defects in iron recycling limit iron availability for erythropoiesis in the bone marrow and cause peripheral iron storage and low serum iron levels similar to what we observe in MACER mice. We hypothesize that ERα signaling facilitates the release of iron from reticuloendothelial macrophages and that macrophage ERα deletion inhibits erythropoiesis and causes anemia. In conclusion, our data support a novel role for macrophage ERα in iron metabolism and systemic iron homeostasis.

The dissertation of Amy Helene Fluitt is approved.

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Curriculum Vitae

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Peer Reviewed Publications

- Williams, K.J., Argus, J.P., Zhu, Y., Wilks, M.Q., Marbois, B.N., York, A.G., Kidani, Y., Pourzia, A.L., Akhavan, D., Lisiero, D.N., Komisopoulou, E., **Henkin, A.H.**, Soto, H., Chamberlain, B.T., Vergnes, L., Jung, M.E., Torres, J.Z., Liau, L.M., Christofk, H.R., Prins, R.M., Mischel, P.S., -Reue, K., Graeber, T.G., Bensinger, S.J. 2013. An essential requirement for the SCAP/SREBP signaling axis to protect cancer cells from lipotoxicity. *Cancer Research* 73:2850-62.
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Patents

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Introduction

Systemic and cellular regulation of iron homeostasis

a. Strict maintenance of iron homeostasis is critical for cell function

Iron is an essential cofactor for cellular metabolic processes ranging from hemoglobin oxygen delivery to the synthesis proteins for the electron transport chain [1]. Tight regulation of iron homeostasis is necessary to prevent oxidative damage because free Fe II iron can participate in redox reactions [2]. On the other hand, iron scarcity limits protein synthesis, which is particularly harmful to mitochondria [3]. Perturbations in iron homeostasis are thought to contribute to the manifestation of many pathologic conditions including diabetes, obesity, and cancer [4-6]. Iron storage diseases are relatively common and arise from mutations in iron-regulatory proteins [7]. Type I hereditary hemochromatosis is by far the most common iron storage disease. Ten percent of Celtic, British, and Scandinavian populations are carriers for the causative mutation in the human hemochromatosis protein (HFE). HFE mutations disrupt serum iron homeostasis and lead to abnormally high dietary iron absorption and accumulation of iron in peripheral tissues. Secondary hemochromatosis can co-occur with hereditary anemias, such as beta-thalassemia and sickle cell anemia, which also lead to dietary iron absorption and pathologic accumulation of iron as the body attempts to maintain appropriate serum iron levels in the face of anemia. Most iron storage disorders are incurable and the sole treatment is for patients to undergo repeated phlebotomy to reduce iron levels in the body [8]. This therapy is invasive, painful, and largely ineffective due to the perpetual need to remove iron from the body as it re-accumulates. This warrants additional research of iron homeostasis regulation to develop better therapies for this common hereditary disease.

b. Iron homeostasis is fundamentally dependent on macrophage iron recycling

Duodenal enterocytes absorb only 1-2mg of dietary iron [9], yet approximately 20-25mg of iron is required each day for hemoglobin synthesis during erythropoiesis. Thus, iron must be recycled to maintain iron homeostasis. Two million senescent erythrocytes are phagocytosed every minute by macrophages in the spleen and liver, effectively recycling 25mg of iron back into circulation to support erythropoiesis [10]. Red pulp macrophages in the spleen clear the majority of senescent erythrocytes and tissue resident macrophages of the liver (Kupffer cells) also contribute to this process. During erythrophagocytosis, senescent RBCs express specific markers to trigger erythrophagocytosis by reticuloendothelial macrophages. Upon phagocytosis of erythrocytes, hemoglobin is broken down into heme by hemopexin in the cytosol and is further metabolized by the hemoxygenase HMOX1 to iron, bilirubin, and CO2 [11-12]. Cytosolic iron is referred to as the labile iron pool (LIP) and the size of this pool must be strictly regulated to prevent Fe II from participating in redox reactions. The majority of the labile iron pool is shuttled to mitochondria for protein synthesis [13]. Excess iron is either stored as ferritin or released into the bloodstream by ferroportin to be used for erythropoiesis in the bone marrow. FPN is the only known iron exporter and it is robustly expressed by red pulp macrophages, Kupffer cells, and hepatocytes [14-15]. Macrophages also take up iron by transferrin-mediated endocytosis. Transferrin receptor 1 (TfR1) recognizes transferrin-bound Fe II and the complex is endocytosed, where acidification of endosomes releases iron. STEAP3 metalloreductase activity reduces Fe II to Fe III for transport into the cytosol by the metal transporter DMT1 [16]. TfR1, DMT1, ferritin, and ferroportin expression are all regulated by intracellular iron levels. IRP1 and IRP2 are iron-sensitive proteins that bind Iron response elements (IREs) under iron deficiency to stabilize Tfr1 and Dmt1 transcripts and to block translation of ferritin and ferroportin [17]. Combined, these actions facilitate iron uptake. Under sufficient or high iron conditions, IRP1/IRP2 do not bind mRNA and ferroportin protein is translated to allow iron efflux from macrophages into the bloodstream.

c. Hepcidin is the master regulator of serum iron homeostasis

The recent discovery of the iron-regulatory hormone, hepcidin, led to a number of important revelations about the mechanisms controlling serum iron homeostasis [18]. Hepcidin is a hormone secreted by hepatocytes which functions to maintain homeostatic serum iron levels. When serum iron levels are sufficient, hepcidin is expressed and binds ferroportin to inhibit iron release by both enterocytes and macrophages [19]. Conversely, when serum iron is low, hepcidin is not secreted does not prevent iron export via ferroportin. Hepcidin production by hepatocytes is regulated by a number of signals, including iron, inflammation, and erythropoietic signals. Transferrin receptors TFR1 and TFR2, along with the hemochromatosis protein, HFE, recognize extracellular holotransferrin concentrations and induce the expression of hepcidin when serum iron is sufficient via unknown mechanisms [20]. Hepatocytes are also thought to induce hepcidin expression in response to intracellular iron concentrations through BMP-SMAD signaling pathways [21]. Failure to produce hepcidin due to mutations in hepcidin or its regulatory proteins results in hereditary hemochromatosis due to the hyper absorption of dietary iron. Erythropoietic signals and hypoxia negatively regulate hepcidin because of the need for additional iron to synthesize erythroblasts [22]. Importantly, hepcidin appears to be functionally equivalent in humans and mice, thus murine studies of iron metabolism have the potential to be directly translatable to humans [23].

d. Iron is at the interface of infection and immunity

Hepcidin is structurally similar to certain antimicrobial peptides and mediates the hypoferremic response during infection and chronic inflammatory diseases [23]. Hepatocytes respond to IL-6 secretion from pro-inflammatory macrophages by synthesizing hepcidin, which results in a rapid decrease in serum iron due to ferroportin blockade on enterocytes and macrophages. This serves to limit the availability of iron to extracellular, iron-dependent pathogens that may be

present in the blood. Iron is essential for microbial life, where it is also used as a cofactor in proteins and as a catalyst in metabolic processes. Pathogens have multiple methods to scavenge iron from their environment. Microbial pathogenesis is tightly linked to the ability of microbes to acquire iron, therefore macrophages must be able to sequester iron away from engulfed pathogens. To do so, macrophages express natural resistance-associated macrophage protein (NRAMP1) on phagosomal membranes in response to inflammatory stimuli and phagocytosis [24].

2. The case for estrogen regulation of iron metabolism

a. Iron homeostasis is sexually dimporhic

Iron deficiency anemia is common in young women due to iron loss during menstruation [25]. Women from the age of adolescence to menopause have lower serum iron levels compared to men, and upon menopause, total iron levels in women increase to that of similarly aged men [26]. Accordingly, pre-menopausal women have lower serum hepcidin levels than men, and oral contraceptives are also thought to negatively regulate hepatic hepcidin expression and contribute to increase iron stores in contraceptive users [27]. Since loss of estrogen signaling equates female iron levels to that of men, there is speculation that estrogen controls iron homeostasis in women. Despite vast clinical evidence, mechanisms for sex hormone regulation of iron homeostasis remain a mystery and continued investigation is needed.

b. Estrogen signaling is implicated in the regulation of hepcidin expression:

In canonical estrogen signaling, estrogens bind ERα and the complex enters the nucleus and binds estrogen response elements to regulate gene expression. ERα can also tether to other transcription factor complexes that include NFKB, SP1, AP1, C/EBP beta. A third mechanism for estrogen signaling involves the G-coupled protein receptor, GPR30, which is responsible for the rapid, non-genomic effects of estrogen that lead to calcium mobilization, cAMP production, PLC activation, and the action of protein kinase cascades such as PI3K/AKT and ERK. ERα and

GPR30 signaling can be both synergistic and antagonistic, thus complicating the study of estrogen-related signaling mechanisms [28].

Three recent studies implicate estrogen, ERα, and the membrane-bound estrogen receptor, GPR30, in the regulation of hepcidin expression. Independent work conducted by Yang [29] and Hou [30] utilized a combination of chromatin immunoprecipitation, EMSA, and luciferase reporter systems to validate the presence of functional estrogen response elements in the hepcidin promoter. Hou also examined hepcidin and iron parameters in ovariectomized (OVX) mice, which is a model used to study postmenopausal changes that occur when estrogen secretion from the ovaries declines. Surprisingly, iron metabolism has not been thoroughly investigated in this model. OVX mice had elevated hepatic hepcidin expression and significantly increased iron stores in the liver and spleen. OVX mice also had small, non-significant reductions in serum iron and hemoglobin. These results suggest that loss of estrogen signaling alters iron homeostasis in mice.

In contrast, a third study claimed that estrogen induces hepcidin via activation of the membrane-associated estrogen receptor, GRP30 [Ikeda, 31]. Paradoxically, both estrogen and the ERα inhibitor, ICI 1827720, function as agonists for GPR30. HepG2 cells treated with either estrogen, ICI 1927720, or a GPR30 agonists all increased hepcidin expression via a GPR30-BMP6 dependent mechanism. In this study, OVX mice had decreased liver hepcidin expression and increased serum and tissue iron. Surprisingly, the Ikeda and Yang studies utilized identical cell lines, culture conditions and nearly identical qPCR primers to detect hepcidin expression. Differences in the length of time between ovariectomy and data collection may partially explain the conflicting iron and hepcidin measurements. It is worth noting that the ranges of serum and tissue iron levels reported in the Ikeda study are outside of the physiological range. It is difficult to interrogate discrepancies between these studies without further knowledge of the experimental details. Clearly, additional work is required to define mechanisms for regulation of hepcidin and iron homeostasis by estrogen, ERα, and GPR30.

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CHAPTER ONE: A SYSTEMS GENETICS APPROACH TO INVESTIGATE LINKS BETWEEN ERα AND IRON METABOLISM BY MACROPHAGES

Introduction

Introduction to mouse genome wide association

Complex traits are inherently difficult to study, but the use of human genome wide association studies has thus far led to the discovery of hundreds of genes that contribute to complex traits such as blood pressure and blood cholesterol levels [1-2]. In genome-wide association studies (GWAS), the variant most strongly associated at a particular locus is presumed to be in linkage disequilibrium with the causal, functional variant. Follow up studies require mapping and deep re-sequencing of the associated loci followed by the application of a number of bioinformatics tools to hone in on the causative variant. A sobering fact about human GWAS is that causative variants usually explain just a small fraction of the total phenotypic variance contributing to the trait being studied [3]. The mouse is a simplified model organism for which association-based approaches can be applied for the study of complex human diseases. Mouse GWAS overcome some of the deficiencies of human GWAS because just a few hundred mice can usually explain more than 50% of the phenotypic variance of a trait [4]. Access to tissues and environmental controls also makes the mouse an ideal model for follow up analysis of genes of interest (GOIs). The unfortunate tradeoff is that mouse GWAS lack the mapping resolution of human studies resulting in an average mapping size of 2Mb, which can include hundreds of GOIs per loci and confound the identification of causative genes [5].

New methods allow for high resolution mapping using mouse GWAS

Developments over the past 5 years have improved the statistical power and mapping resolution of mouse GWAS studies and accelerated their use in the evaluation of complex traits implicated in human disease [6-9]. The extensive population structure that results in over-

estimations of predicted associations in mice can now be corrected for with efficient mixed-model association (EMMA) [6]. However, the statistical power of associated variants is very disappointing after population structure correction because of the poor mapping resolution of most mouse association studies. To address this, *Bennet, et al*., devised a panel of 100 classic and recombinant inbred mouse strains (Hybrid Mouse Diversity Panel, HMDP) that retains the statistical power to map traits after correction for population structure [7]. Using natural genetic polymorphisms between mouse strains, differences in the expression of specific genes between individuals can be mapped to SNPs that correlate with or influence the expression of another gene. The HMDP has since successfully mapped traits at a resolution of less than 1Mb, which is an order of magnitude higher than what was previously achieved in mouse associations [8-9]. In addition, the genomes of mice in the HMDP are all sequenced and this eliminates the need for re-sequencing and genotyping during follow up studies on candidate loci. These improvements in mouse GWAS should streamline the connections between loci and disease pathway and identify promising new targets with implications in human disease.

The Systems Genetics Resource

The Systems Genetics Resource (SGR) is a publicly available database of genetic mapping data from mouse and human populations to aid researchers in the study of complex traits [10]. One of the most valuable tools made available through the SGR is the hi-resolution association-mapping data of the Hybrid Mouse Diversity Panel (HMDP). The datasets are the result of tissue expression profiling of the hybrid mouse diversity panel (HMDP) acquired with Affymetric Mouse Genome HT_MG-430A arrays. The SGR enables users to quickly mine HMDP datasets for expression quantitative trait loci (eQTL), which are genomic locations associated with the expression of a gene of interest. eQTLs are enriched for miRNA binding sites, splice enhancer/ silencer sites, transcription factor binding sites, non synonymous mutations, and copy number polymorphisms [11]. Additionally, users can search for genes that are correlated with a gene of

interest. The following chapter describes our efforts to uncover novel functions of ERα in macrophages using the SGR.

Methods

Mining the Systems Genetics Resource for genetic relationships of Esr1 in macrophages

We input Esr1 in the Systems Genetics Resource (SGR, http://systems.genetics.ucla.edu) search queries for expression quantitative trait loci (eQTL) and gene correlations. We searched for eQTL in the Hybrid Mouse Diversity Panel (HMDP) macrophage dataset, and we searched for gene correlations in both macrophages, bone, and liver. We included bone and liver because macrophages are resident cells in these tissues and thus contribute to the expression profiles. In our eQTL analysis, we identified all annotated genes within 1 megabase of each peak SNP identified by the database because these genes are in linkage disequilibrium with the SNP. We used publicly available gene expression databases (e.g. Immgen, BioGPS) to estimate the expression of these genes in macrophages, and for genes known to be expressed by macrophages, we used published qPCR primer sequences to examine their expression in shEsr1 and shScr RAW macrophages. For our *Esr1* correlation analysis, we downloaded pre-computed tables of genes correlated to *Esr1* from the SGR and searched for genes ($r > .3$) related to immune function.

Estrogen response element query

The Estrogen Response Element Finder is a resource developed and opERαted by the Genome Institute of Singapore, Institute for Infocomm Research, and the Karolinska Institute. (http://datam.i2r.a-star.edu.sg/ereV3/). We input mouse FASTA sequences for genes of interest to obtain the number of potential estrogen response elements (EREs) within each gene sequence (Table 2.3).

Cell culture

We used stable shScr and shEsr1 RAW 264.7 cells were cultured in DMEM + 10% FBS under puromycin selection. For experiments, cells were plated at a density of 250,000 cells/well in 24-

well plates in triplicate and treated the following day with 1ug/mL LPS for a total of 24 hours before lysing cells in TriZol reagent for RNA isolation.

Quantitative PCR:

RNA was extracted using TriZol reagent and RNeasy columns (Qiagen). cDNA synthesis was performed using 1µg of RNA with SuperScript II reverse transcriptase (Invitrogen). Gene expression analysis was performed on a BioRad MyiQ real time detection system using iQ SYBER Green Supermix (BioRad). Genes are expressed as relative mRNA level compared with the housekeeping gene Ppia. Primer pairs were used based on published sequences.

Bone marrow macrophage differentiation

Both femurs and tibias from a wild type C57Bl/6 female mouse were flushed with MACS buffer (PBS + 2% FBS and 1mM EDTA). After RBC lysis, bone marrow cells were counted on a hemacytometer and plated in L929 medium (DMEM+ 10% FBS + 30% L929 conditioned media) at a density of 10^6 cells/mL in non-TC treated 20cm plates. Media was refreshed on day 3 of differentiation and day 7 mature macrophages were plated in triplicate at the same density into 6-well plates for experiments. Cells were treated with 10nM 17beta estradiol (E2) for 24 hours before lysing cells in TriZol reagent for RNA isolation.

Results

Evaluation of Esr1 eQTL hotspots

We queried the Systems Genetics Resource for eQTLs associated with *Esr1* expression in basal, LPS, and OxPAPC-stimulated macrophages. No SNPs met threshold for genome-wide significance (LOD=5.4), however there were three SNPs on the cusp of this threshold (Table 1.1) Of the genes located in close proximity to the SNPs, the Metallothionein family (MT1-4) was particularly interesting because MT1 is highly expressed by macrophages and is known to be essential for macrophage function (ImmGen.org). Metallothioneins are metal binding proteins that scavenge free radicals and MT1 null mice have defective phagocytosis and antigen presentation [12]. Metallothioneins were not highly expressed by any other hematopoietic lineage except Langerhans dendritic cells (ImmGen.org), implying that MT1 may perform an important function that is specific for macrophage function. We next queried the Systems Genetics Resource for genes correlated with *Esr1* in macrophages and identified genes related to cytokine signaling, chemotaxis, and cellular metabolism (Table 1.2). Notably, *Esr1* correlated with the potential eQTL, Mt1. Macrophage *Esr1* also correlated with STEAP3, a metalloreductase that is critical for macrophage iron metabolism. We next searched for *Esr1* correlations in liver and bone because macrophages function within these tissues to regulate iron homeostasis. In the liver, *Esr1* correlates with *Hamp1*, which encodes the master regulator of iron metabolism, hepcidin. Recent studies show that estrogen/ERα signaling can both positively and negatively regulate hepcidin expression. The verified correlation between ERα and *Hamp1* in the liver gives us confidence in our systems approach to study the relationship between ERα and iron homeostasis. In total, *Esr1* correlated with 10 different iron-related proteins across macrophages bone, and liver datasets. Considering these findings, we asked whether iron metabolism genes contain estrogen response elements (EREs). We were surprised to find that out of 25 critical iron metabolism genes, 80% have EREs and the average number of EREs is 5.5 per gene (Table 1.3). Some genes contained many EREs: *Aco1* (10),

Aco2 (10), *Exoc6* (29), and *Steap3* (15). While a single ERE can be sufficient for ERα to bind and regulate transcription, multiple EREs increase ERα binding capability and the likelihood that ERα utilizes these regulatory elements in vivo.

In vitro evaluation of iron metabolism genes in shEsr1 RAW macrophages:

Table 1.1 eQTL loci and associated genes for Esr1 in macorphages

Condition	SNP ID	P value	LOD Score	Local Genes
Basal	rs13479927	9.74E-06	5.01145442	*Gnao1,* ***Mt1-4****, Ogfod1, Nup93, Nud21*
LPS	rs6253968	7.24E-06	5.14021944(	*Kcnq5, Il17a/b, Mcm3, Paqr8, Efhc1, Tmem14a, Gsta3*
OxPAPC	rs29229611	7.24E-06	5.14021944(	*Elmo1, Tcrg, Gpx5, Aoah, Stard3nl, Nme8, Gpr141, Trim27, Cdk13, Rala, Pouf6f2, Amph, Vsp41, Yae1a, Rhag*

Table 1.2 Genes correlated with Esr1

Tissue	Gene	Corr. Coef. (biweight mid correlation)
Macrophage	*Mt1*	0.334
	Steap3	0.347
Liver	*Slc11a2/ Dmt1*	0.715
	Hamp1	0.794
	Tmprss11f	0.776
	Tmprss3	0.722
	Aco2	0.606
Bone	*Aco2*	-0.345
	Aco1	-0.42
	Tmprss11c	0.46
	Frataxin	0.49

Table 1.3: Potential EREs in the promoter regions of iron metabolism-related genes

Gene	Function	Potential ERE's
Abcb7	iron-sulfur cluster assembly, heme synthesis	9
Aco1/Irp2/ Irebp2	iron response element binding protein	10
Aco2	mitochondiral iron metabolism	10
Bmp2	regulates hepcidin expression	3
Ceruloplasmin	plasma iron transport	0
Dcytb/Cybrd1	duodenal and macrophage iron absorption	3
Dmt1/SLC11A2	dietary iron absorption, transferrin cycle	8
Exoc6	intracellular iron transport	29
Ferrochelatase	heme synthesis	10
Ferritin	intracellular iron storage	0
Ferroportin/ SLC40A1	iron export	2
Frataxin	iron-sulfur cluster assembly	0
Frrs1	iron transport from endosome to cytosol	0
Furin	hepcidin activation	2
Hmox1	heme catabolism into bilirubin	2
Hrg1/ SLC48A1	heme transport	3
Metallothion ein	metal binding and transport	3
Nramp1/ SLC11A1	phagosomal iron transport	3
Pcb2	iron chaperone	4
Steap3	endosomal iron transport	15
Tfrc	iron uptake	0
Tfr2	cellular iron uptake	3
Tmprss	blocks hepcidin	6
Zip14/ SLC39A14	transferrin independent iron uptake	3

We next evaluated the expression of the potential eQTL, Mt1/Metallothionein, as well as iron metabolism genes correlated to *Esr1* in shEsr1 RAW 264.7 macrophages. There was no change in *Mt1* expression in shEsr1 compared to shScr macrophages, but both *Steap3* and *Irp1* expression were significantly elevated in shEsr1 cells (Fig 2.1a). STEAP3 is a metalloreductase

that converts insoluble ferric iron (Fe^{3+}) to the soluble ferrous (Fe^{2+}) form and is required for efficient iron transport by the endosome following transferrin-mediated iron uptake [13]. IRP1 (and IRP2) are critical for the post-transcription regulation of cellular iron homeostasis. IRP1 and IRP2 are iron-sensitive proteins that bind iron response elements (IREs) on mRNAs in iron-depleted cells to inhibit the translation of ferritin (iron storage), ferroportin (iron export) and to increase the stability of TfR1 (iron uptake) mRNA [14]. Together, these changes function to increase the labile iron pool for utilization by mitochondria in the synthesis of iron-sulfur-containing proteins and other metalation reactions. When we examined an additional panel of macrophage iron metabolism genes, we found small, but significant differences in the expression of genes encoding iron uptake (*Tfr1, Ngal-R, Cerulloplasmin*), storage (*ferritin L*), transport (*Exoc6*) and export (f*erroportin)* (Fig 2.1b). Cellular iron levels are acutely maintained to avoid oxidative stress, therefore even subtle changes can have a significant impact on macrophage iron metabolism.

Iron metabolism genes respond to estrogen in female bone marrow macrophages

RAW 264.7 macrophages cannot accurately predict the expression profile of primary macrophages and generally have lower expression of all iron metabolism genes compared to isolated murine macrophage populations (BioGPS). RAW 264.7 macrophages are derived from a male mouse, which can also confound the study of estrogen/ERα signaling if ERα functions in a sexually dimorphic manner. To address these limitations, we derived bone marrow macrophages from wild type female mice and examined changes in iron gene expression in response to overnight estrogen (17-beta estradiol, E2) treatment. Upon estrogen stimulation, the expression of endolysosomal iron transporters *Steap3, Dmt1a, Dmt1a,* and *Nramp1* were all reduced (Fig 2.1c). The expression of the only known iron exporter, ferroportin, was also repressed by estrogen. Iron and heme metabolism are closely related and we saw that estrogen treatment represses *Hmox1* (heme catabolism) and induces *Fech* (heme synthesis), thus favoring heme synthesis.

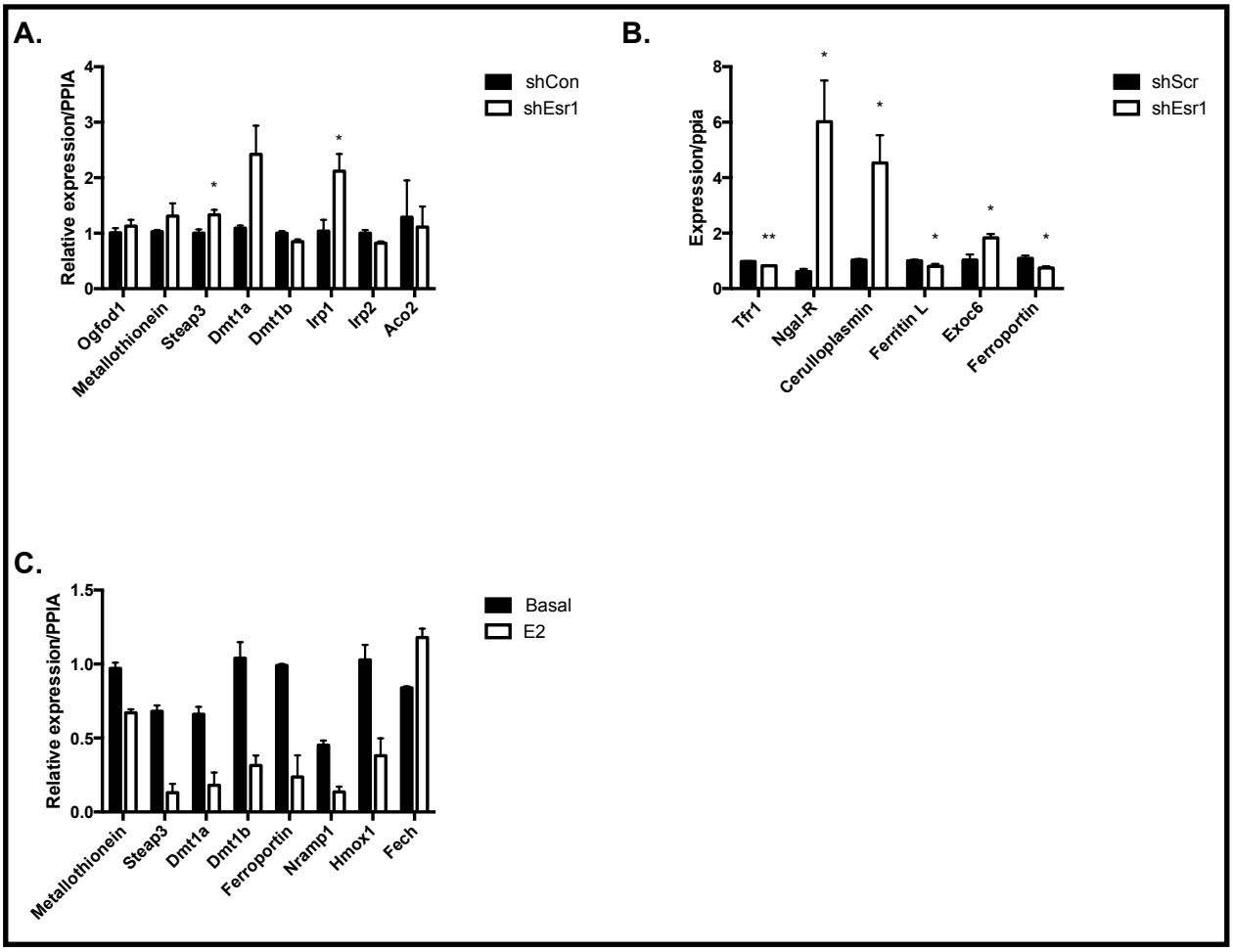

Figure 2.1: Iron metabolism gene expression in shEsr1 RAW 264.7 macrophages and primary macrophages

A-B. Gene expression in shCon and shESr1 RAW 264.7 macrophages. Error bars indicate mean +/- SEM. n=3 wells per group. C. Gene expression in primary female macrophages treated with 10nM E2 overnight on day 8 post-differentiation. Error bars indicate mean +/- SEM. n=3 wells per group

Discussion:

Estrogen signaling mediated by ERα exerts pleiotropic effects on the biology of macrophages. As expected, our query for *Esr1* in the Systems Genetics Resource revealed genetic links between a number of critical, macrophage-specific genes and the expression of ERα. We identified several regions of the genome suggested to regulate ERα expression, but the surrounding genes did not encode transcription factors or proteins with known regulatory functions relevant to ERα signaling. Many eQTL are difficult to interpret because they are miRNA binding sites and splice-accepting sites. We instead focused our search on genes that correlate with ERα in tissues essential for iron metabolism (macrophages, liver, bone). Of note, ERα correlates with metallothionein1 (MT1), which is also located in an eQTL regions for *Esr1*. MT1 participates in metal detoxification and MT1 deletion results in numerous macrophage defects [12]. The function of MT1 is not entirely clear so we cannot predict how MT1 might regulate ERα expression as an eQTL. *Esr1* also correlates with the metalloreductase, *Steap3*, in macrophages, which has a well defined role in cellular iron metabolism. When Steap3 -/- macrophages undergo erythrophagocytosis, iron becomes trapped in the endosomes and lysosomes due to the inability to reduce Fe III to Fe II for transport into the cytosol [13]. Negative regulation of *Steap3* is also important for the hypoferremic response of infection to sequester iron within macrophages. We believe it is possible that ERα is a novel, negative regulator of *Steap3* because expression of this gene increases upon ERα deletion.

The genetic link between *Esr1* and iron metabolism was further solidified by our observation that virtually all iron-related genes have multiple estrogen response elements where ERα can bind and regulate transcription. When we evaluated iron gene expression in shEsr1 macrophages, we found small, but significant differences in the expression of genes related to iron uptake, storage, endosomal transport, and export. Overnight estrogen treatment significantly reduced the expression of most iron-related genes in bone marrow macrophages, suggesting that

estrogen/ERα signaling may function to negatively regulate iron metabolism. This hypothesis is supported by the fact that iron accumulation occurs in postmenopausal women after the decline in estrogen production. Together, our results show that ERα is genetically linked to iron metabolism in macrophages, bone, and liver, and that estrogen/ERα can regulate iron metabolism in female bone marrow macrophages. This is an important finding because very little is known about the role of estrogen signaling and macrophage iron metabolism despite enormous speculation that estrogen regulates systemic iron homeostasis in women.

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CHAPTER TWO: MACROPHAGE-SPECIFIC ERα DELETION IMPAIRS IRON RELEASE FROM RETICULOENDOTHELIAL STORES

Introduction

In the previous chapter, we strengthened our hypothesis that ERα plays a role in macrophage iron homeostasis by identifying genetic links between the expression of *Esr1* and genes that regulate iron homeostasis. To determine if ERα is required for macrophage iron homeostasis, we utilized a mouse model of myeloid-specific ERα deletion (MACER). Our group previously utilized the MACER mouse model to investigate the role of macrophage ERα in inflammation and metabolic homeostasis [1]. In this study, we found that ERα-deficient macrophages have impaired alternative activation in response to IL-4. MACER macrophages had numerous other defects involving phagocytosis, metabolic parameters, and inflammatory cytokine production, all of which influence iron homeostasis. In the current chapter, we investigate whether MACER mice have defects in iron metabolism and homeostasis.

Methods:

Animals: Esr1 flox/flox mice [2-3] were crossed into a transgenic line where Cre recombinase is driven by the lysozyme M promoter to generate myeloid-specific ERαKO mice (named MACER mice). Mice were obtained by JAX. Age/strain matched *Esr1*$^{flox/flox}$ and LysM Cre female mice were used as controls. Mice were maintained in pathogen-free facilities at the University of California, Los Angeles (UCLA). All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Animal Research Committee of UCLA. Experiments were performed on animals 2-3 months in age unless specified otherwise.

Complete blood counts

Blood samples were collected at time of animal sacrifice and CBC analyses were performed on a Heska HEMAVET and provided by DLAM.

Serum Iron Analysis: Mouse blood was collected from the posterior vena cava and centrifuged for 5min at 4500rpm. Supernatant was removed, centrifuged again, and the new supernatant was put in a fresh tube and frozen at -80°C for storage before iron analysis. In a 96-well assay plate, 10uL serum from each mouse was plated in three or more replicate wells. Serum iron content was determined with the colorimetric Iron-SL Kit (Sekisui) by adding 150uL acid dissociating buffer and 40uL color reagent to each well. Plates were read at 595nm after a 5 minute incubation and iron concentration was determined using a multi-point standard curve.

Tissue Iron Analysis: A small piece of flash frozen liver from each mouse was weighed in a pre-tared microcentrifuge tube and homogenized in 1125uL protein precipitation buffer (.53N HCL and 5.3% trichloroacetic acid in HPLC water). Samples were boiled for 1 hour and then

centrifuged for 10 minutes at maximum speed. The supernatant was recovered and stored at -80°C for iron analysis. 30uL of the tissue supernatant was used in each well and the iron assay was performed the same as with serum.

Serum and Tissue Heme Analysis: Serum and tissue samples were prepared the same as for iron analysis. Sample heme concentration was determined using the colorimetric Quantichrome Heme Assay Kit (Bioassay Systems). 25uL of either serum or tissue supernatant were assayed in triplicate after a 5min incubation in 200uL heme kit reagent. Absorbance was read at 400nm and heme concentration was calculated with a multi-point standard curve.

Histology.

Perls Prussian Blue Stain for Iron: Spleen and liver sections were deparaffinized in xylenes through ethanol before staining with Perls prussian blue stain for iron per manufactures instructions (Sigma). Slides were analyzed on a Nikon Eclipse 90i slide scope.

H&E stain: Paraffin blocks of liver and spleen were submitted to UCLA's Translational Pathology Core Laboratory (TPCL) for staining.

F4/80 stain: Paraffin blocks of spleen were submitted to UCLA's Translational Pathology Core Laboratory (TPCL) for staining.

Quantitative PCR

RNA was extracted using TriZol reagent and RNeasy columns (Qiagen). cDNA synthesis was performed using 1µg of RNA with SuperScript II reverse transcriptase (Invitrogen). Gene expression analysis was performed on a BioRad MyiQ real time detection system using iQ SYBER Green Supermix (BioRad). Genes are expressed as relative mRNA level compared with the housekeeping gene *Ppia*. Primer pairs were used based on published sequences. When

indicated, datasets from separate cohorts/experiments were combined by normalizing the expression of control animals to 1.

Cell Culture

Both femurs and tibias from a wild type C57Bl/6 female mouse were flushed with MACS buffer (PBS + 2% FBS and 1mM EDTA). After RBC lysis, bone marrow cells were counted on a hemacytometer and plated in L929 medium (DMEM+ 10% FBS + 30% L929 conditioned media) at a density of 10^6 cells/mL in non-TC treated 20cm plates. Media was refreshed on day 3 of differentiation and day 7 mature macrophages were plated in triplicate at the same density into 6-well plates for experiments. For treatments, cells were treated with 10nM estrogen (E2) for 24 hours before lysing cells in TriZol reagent for RNA isolation. When noted, cells were cultured in phenol-red free media prepared with charcoal-stripped FBS for 48 hours before and during experimental treatments.

Flow Cytometry

Cell surface markers: Single-cell suspensions of day 7 bone marrow macrophages were incubated with Fc block before staining with fluorescently labeled primary antibodies for 30 min at 4 °C. F4/80-allophycocyanin (APC), MHC-class II-PE, CD11c-PE-CY7, and CD11c-Percp-Cy5.5 antibodies were used. Cells were stained for 30 min at 4 °C before, washed 3x's in PBS, and resuspended in 400uL FACS buffer for flow cytometry. For additional analyses, basal or LPS-stimulated day 8 bone marrow macrophages cells were stained with 10 mM DCFDA, 100nM Mitotracker Green, 75nM Lysotracker Red, or 5uM Mitosox (all from LifeTechnologies) for 20 minutes at 37°C and washed 3x's with PBS before resuspension in 400uL FACS buffer. Cells pre-treated with 1uM antimycin A for 15 minutes served as positive controls. Flow cytometry was performed on a LSRII and data was analyzed using FlowJo

LPS Challenge

Mice were injected intraperitoneally with 500ng/kg LPS after baseline blood samples were collected via retro-orbital eye bleeds. Two hours post-injection, animals were sacrificed and a terminal blood sample was drawn to compare serum iron levels before and after LPS treatment.

Results

LysM-cre mediated deletion of Esr1 in macrophages

To study the role of ERα in macrophage iron metabolism, we generated mice that lack Esr1 in the myeloid lineage by crossing $Esr1^{flox/flox}$ mice with a LysM cre strain on the same C57Bl/6 background. In $Esr1^{LysM/LysM}$ mice (referred to as MACER mice), floxed Esr1 is deleted in mature macrophages, monocytes, and granulocytes. At the mRNA level, Esr1 is reduced by approximately 90% in MACER bone marrow macrophages and is unchanged in MACER liver, muscle and adipose tissues, demonstrating that MACER macrophages have the targeted deletion for Esr1 [1].

Disruption of Esr1 in mouse macrophages results in severe anemia

At 3-5 months of age, MACER mice appear phenotypically normal to control mice, with no observed differences in body weight, spleen, or liver weight (2.1a-c). However, whole blood analyses revealed that the frequency and absolute number of lymphocytes in MACER are negatively impacted (2.1d-e) and the red blood cell (RBC) count is dramatically decreased compared to age/sex matched control mice (2f, p=.009). Hematocrit for MACER mice is 26.3% +/- 1.86% (mean +/- SEM, n=3), compared to 42.96% +/- 3.38% for control mice (2.1h, p=.01). Consistent with this finding, MACER mice also have abnormally low mean hemoglobin levels of 7.7g/dL +/- 0.45 g/dL compared to 12.66 ug/dL +/- 0.96 ug/dL in control mice (3.1i, p=.01). A ~40% reduction in RBC count, hematocrit, and hemoglobin indicates severe anemia in MACER mice. Anemias manifest from defects in RBC development and clearance, which are dependent on the actions of resident macrophages in the bone marrow and spleen, respectively. Thus, it appears that ERα expression in macrophages may be required for one or more of the processes that influence circulating RBC numbers.

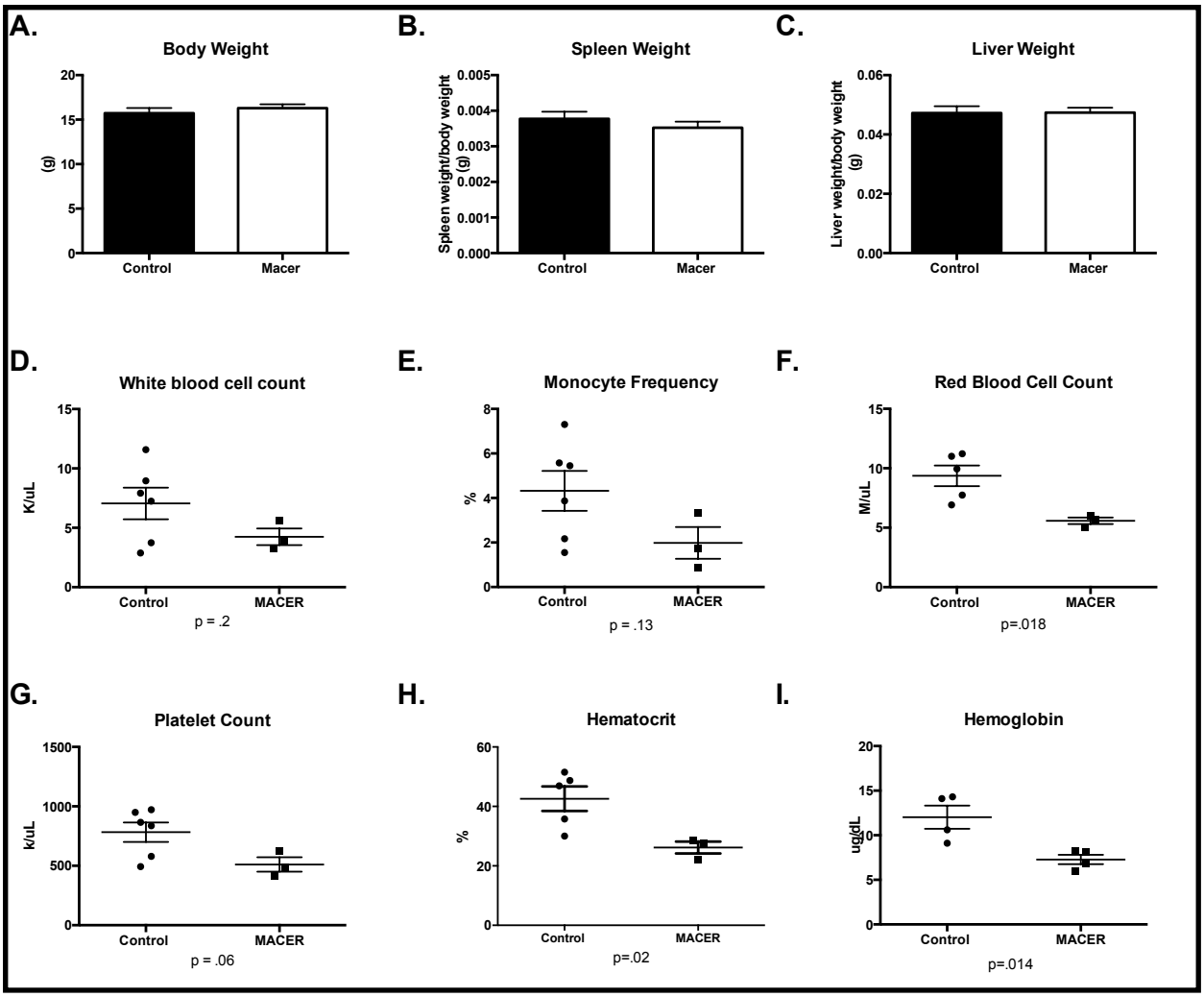

Figure 2.1: **MACER mice are severely anemic**
A-C. Terminal body weight and tissue weights for female Cre+ control and MACER animals (n=3, 2 months of age). D-I. Select parameters from complete blood counts (n=3, 3-5 months of age). Error bars indicate mean +/- SEM.

MACER mice have reduced serum iron coupled with peripheral iron accumulation

To help determine the source of anemia in MACER mice, we measured iron levels in serum and peripheral tissues using a standard colorimetric assay for free iron. MACER mice had lower serum iron levels (236+/-26 ug/dl) compared to control mice (348+/- 29ug/dl, p =.02) (2.2a). When we measured peripheral iron storage and observed elevated levels of iron in the spleen (100% increase, p=.008) and liver (40% increase, p=.01) (2.2b-c) of MACER mice. Macrophages in the spleen and liver phagocytose senescent RBCs and recycle the iron to sustain erythropoiesis and homeostatic RBC levels. Since MACER mice have low circulating iron levels and greater peripheral tissue iron storage than control mice, we hypothesized that MACER mice do not effectively recycle iron from senescent RBCs. H&E stains showed normal spleen physiology, indicating that the level of iron accumulation in MACER mice at 8 weeks is relatively mild and does not appear to cause inflammation, fibrosis, or shrinkage of the red pulp (Fi). While spleen was smaller in size, flow cytometry analysis confirmed that the frequency of different myeloid populations and lymphocytes was the same as in control animals (data not shown). We stained spleen sections with Perls prussian blue and observed iron deposits localizing to the red pulp, which is the region of the spleen where macrophages phagocytose senescent RBCs and recycle iron back into the blood stream (2.3d-e). F4/80 staining of spleen sections was the same between control and MACER mice (2.3f-g). Together, these results suggest that MACER mice have normal numbers of splenic macrophages, but that these macrophages may have defects in iron recycling. We suspect that red pulp macrophages fail to export iron following erythrophagocytosis.

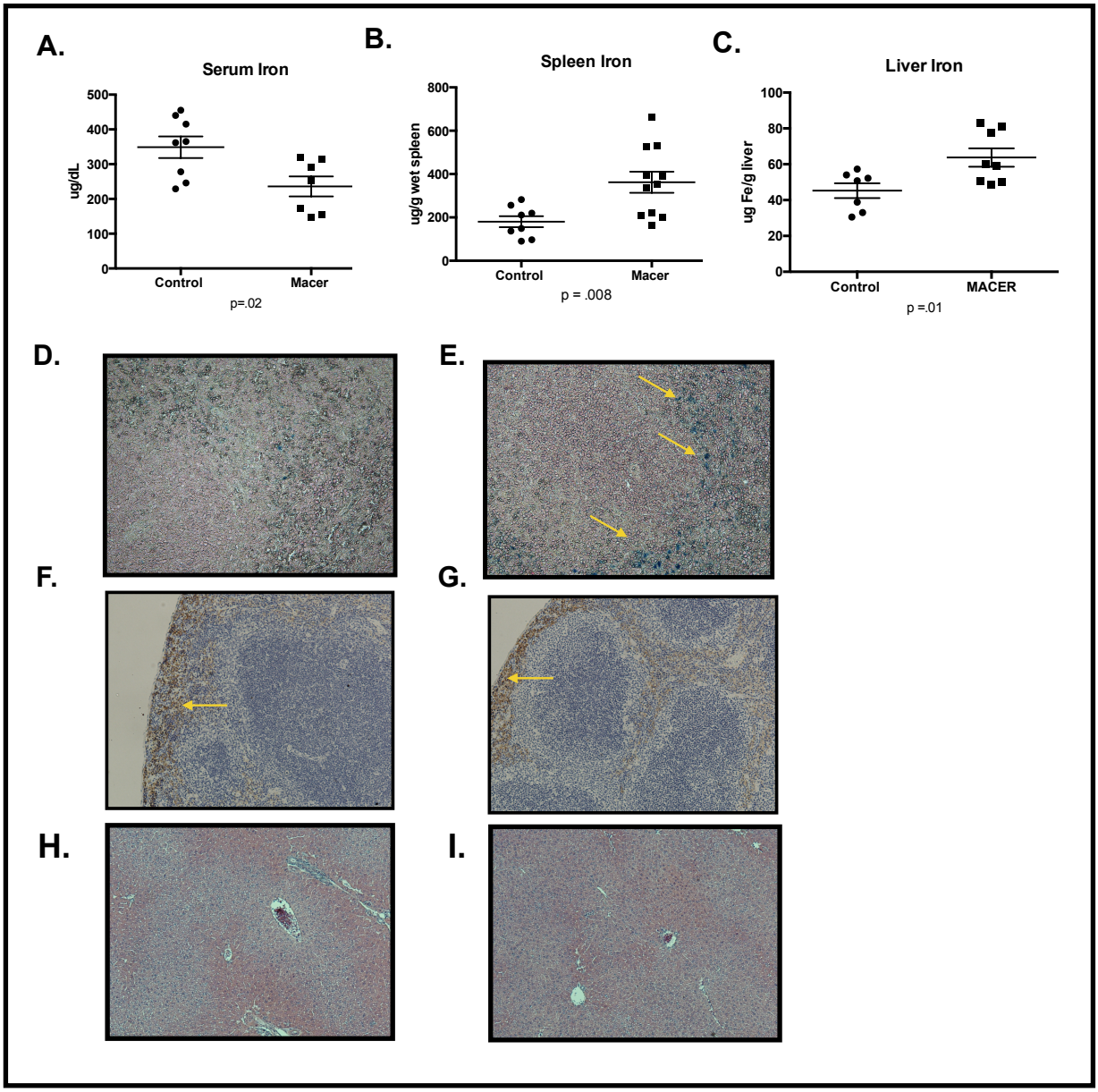

Figure 2.2: MACER mice have low serum iron coupled with peripheral iron accumulation

A-C. Serum iron, liver iron, and spleen iron measurements. Error bars indicate mean +/- SEM. Representative 20x images of Perls prussian blue stain for iron in spleen for D. Cre + control and E. MACER. Yellow arrows indicate iron laden macrophages in the red pulp region. Representative 10x images of F4/80 staining of F. Cre+ control spleen and G. MACER spleen. Yellow areas indicate regions of dark F4/80 staining. Animals were 8 weeks old at the time of tissue harvest. H-I. Representative H&E stains of Cre+ control (H) and MACER (I) liver sections.

The expression profile of MACER splenocytes is consistent with iron overload

Flash frozen MACER spleen had an expression profile consistent with iron overload. The 2-fold increase in total spleen iron observed in MACER mice is most likely responsible for the induction of ferroportin (3.25-fold, p =.02), ferritin (4-fold, p =.01), and hemoxygenase 1 (2.7-fold, p=.008) observed in MACER splenocytes (2.3b-d). Iron and heme induce the expression of all three genes during erythrophagocytosis, therefore we believe that red pulp macrophages from MACER mice are indeed actively phagocytosing erythrocytes. Further support for this includes the slightly elevated expression of the endosomal heme transporter, HRG1, and the unchanged expression of the rate limiting enzyme in heme synthesis, FECH (2.3e-f.) Expression of ferrorportin, ferritin, and hemoxygenase 1 are also essential for macrophages to avoid iron-related toxicity and oxidative stress by enabling storage and export of excess iron and the catabolism of excess heme. This expression profile is consistent with iron retention by MACER splenocytes and suggests that MACER red pulp macrophages actively phagocytose senescent erythrocytes in the spleen. We predict that MACER red pulp macrophages engage in erythrophagocytosis but do not effectively release the recycled iron. Since ferroportin is regulated at the translation level by IRP1/2 and at the protein level by hepcidin, it remains unclear if ferrorportin protein is actively expressed on the membrane of MACER red pulp macrophages. Although expression was low for both genotypes, we detected less *Hamp1* (hepcidin) expression in MACER mice and a small, insignificant increase in a negative regulator of hepcidin, *Tmprss6* (2.3g-h). We predict that circulating hepcidin is low in MACER mice due to their low serum iron and hemoglobin levels. We can confirm this via ELISA for serum hepcidin and/or immunoblot of liver hepcidin expression.

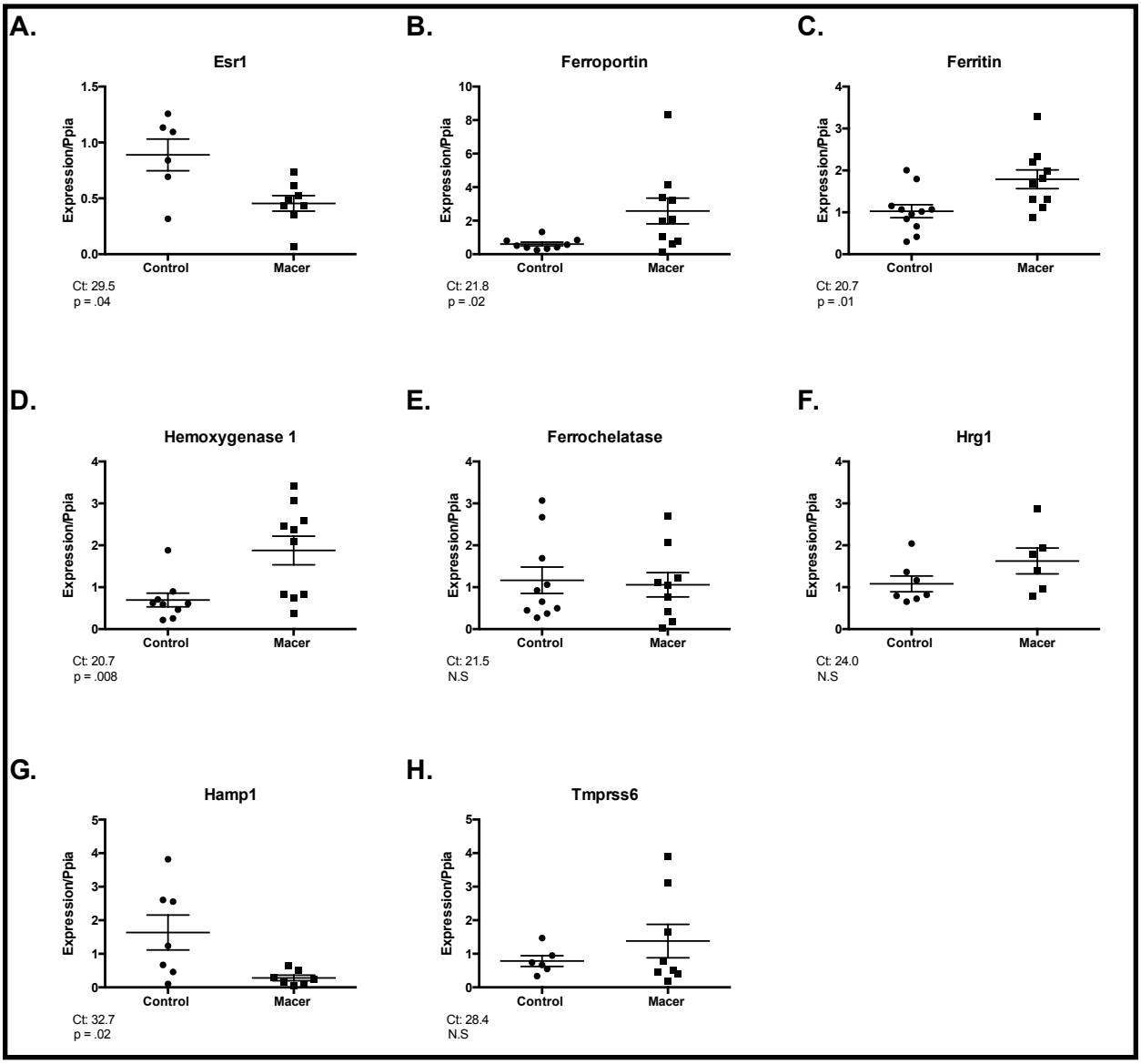

Figure 2.3: **Gene expression in MACER spleens is consistent with iron overload**
A-H: Expression of iron genes in spleens from 8 week old mice. Each dot represents one mouse. Each graph represents combined datasets from two different qPCR plates normalized to control mice set to 1. Error bars indicate mean +/- SEM.

MACER bone marrow macrophages display differences in iron metabolism gene expression

Bone marrow progenitors from both genotypes differentiate into mature bone marrow-derived macrophages (BMMs) after 7 days of culture supplemented with GM-CSF and express similar levels of the macrophage surface markers F4/80, Cd11b, and MHC II (data not shown). MACER BMMs have an 82% reduction in *Esr1* expression (2.4a, p = .005). To investigate whether Esr1-null macrophages have intrinsic defects in iron homeostasis, we performed gene expression analyses on BMMs cultured in phenol red-free medium prepared with charcoal-treated FBS to remove exogenous estrogens. We observed changes in the expression of genes involved in several different steps of iron metabolism in MACER BMMs. *Fech* (2.4b, up 31%, p=.01) is the rate limiting enzyme in heme synthesis located in mitochondria, *Tfr2* (2.4c, up 37%, p =.03) can aid in macrophage iron uptake, and *Nramp1* (2.4d, up 2-fold, p =.04) and *Steap3* (2.4e up 2-fold, p =.04) are located at phagosomal and endolysosomal membranes, respectively, to aid in iron transport and conversion of ferric iron (Fe II) to soluble ferrous iron (Fe III). It is possible that ERα negatively regulates the expression of Nramp1, Steap3, Tfr2, and Fech in red pulp macrophages to influences erythrophagocytosis. These genes also respond to pro-inflammatory signals, so alternatively, changes in pro-inflammatory cytokine expression in ERα-null macrophages could induce changes in expression. Importantly, the iron present in BMM differentiation and maintenance medium (100-200ng Fe/mL) is only a fraction of what is found in the circulation or in peripheral tissue. Iron-deficient conditions activate IRP1/2 to prevent the translation of ferritin and ferroportin and to increase transcription stability of Dmt1 and transferrin receptor 1 (TfR1) to stabilize intracellular iron levels. As expected, there was no difference in the mRNA expression of ferrorportin, ferritin, Dmt1, or TfR1 between MACER and control BMMs (data not shown).

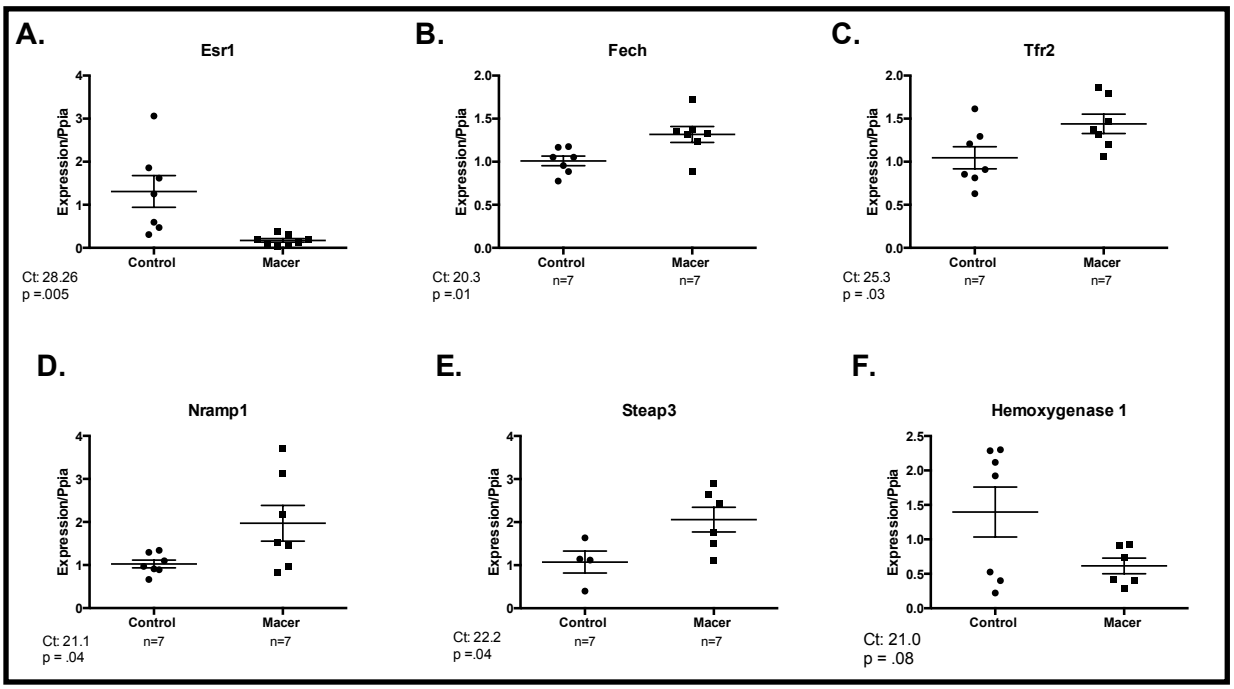

Figure 2.4: **Expression of iron metabolism genes are altered in MACER macrophages**
A-F: Expression of iron genes in day 9 bone marrow macrophages from 8 week old mice. Each dot represents one mouse. Each graph represents combined datasets from two different qPCR plates normalized to control mice set to 1. Error bars indicate mean +/- SEM.

The hypoferremic response is defective in MACER mice

To begin investigating the hypoferremic response in MACER mice, we injected MACER and control mice (n=1) with 500ng/kg LPS and collected serum samples 2 hours post-injection. When we compared serum iron levels before and after LPS administration, we saw that while the floxed *Esr1* control mouse was capable of reducing serum iron levels in response to LPS, the MACER mouse actually experienced a small increase in serum iron (2.5a). It is possible that MACER mice cannot deplete serum iron during infection Change in ferroportin, ferritin, TFR1, and NRAMP1 expression are critical for iron sequestration by macrophages during infection and we speculate that defective signaling in these genes by MACER mice renders them incapable of reducing serum iron levels in response to infection. We must repeat this study with additional animals to confirm whether the hypoferremic response is in fact defective in MACER mice.

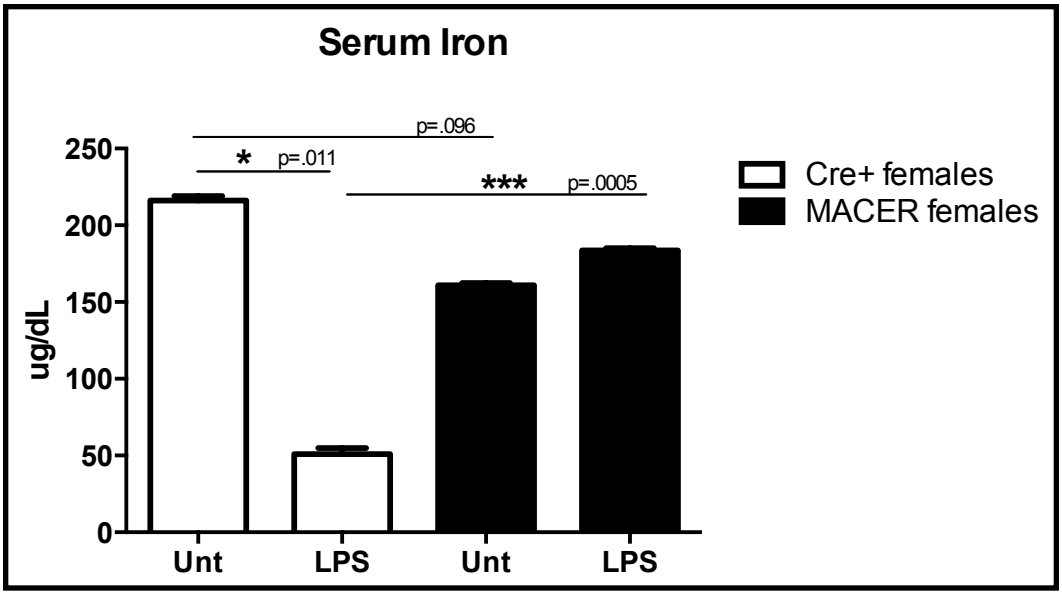

Figure 2.5: **MACER mice fail to sequester iron from serum in response to infection**
Basal serum iron compared to serum iron 2hrs after LPS-injection (500ng/Kg). (n=1). Error bars indicate mean +/- SEM. technical triplicates)

MACER mice compensate for low serum iron by up regulating intestinal iron transporters

Under low serum conditions, hepcidin is not secreted by hepatocytes and therefore ferroportin is capable of releasing iron from enterocytes in the duodenum and from macrophages to help stabilize serum iron homeostasis. The duodenal iron uptake protein, DCYTB, in addition to iron transporters DMT1, and ferroportin are also transcriptionally regulated by low iron and hypoxia to increase duodenal dietary iron uptake and the release of iron into the bloodstream. We examined duodenal gene expression in control and MACER mice (n=1) to gather preliminary data regarding dietary iron absorption. Consistent with the reduced serum iron in MACER mice, both Dcytb and Dmt1a are significantly up regulated in MACER intestine (2.6a). Dmt1a contains an IRE, but Dmt1b does not. Ferroportin expression is also much higher in MACER intestine, which can aid in the release of iron from enterocytes into the blood stream. Further investigation with additional mice will help determine if and how MACER mice attempt to compensate for low serum iron by increasing dietary iron absorption.

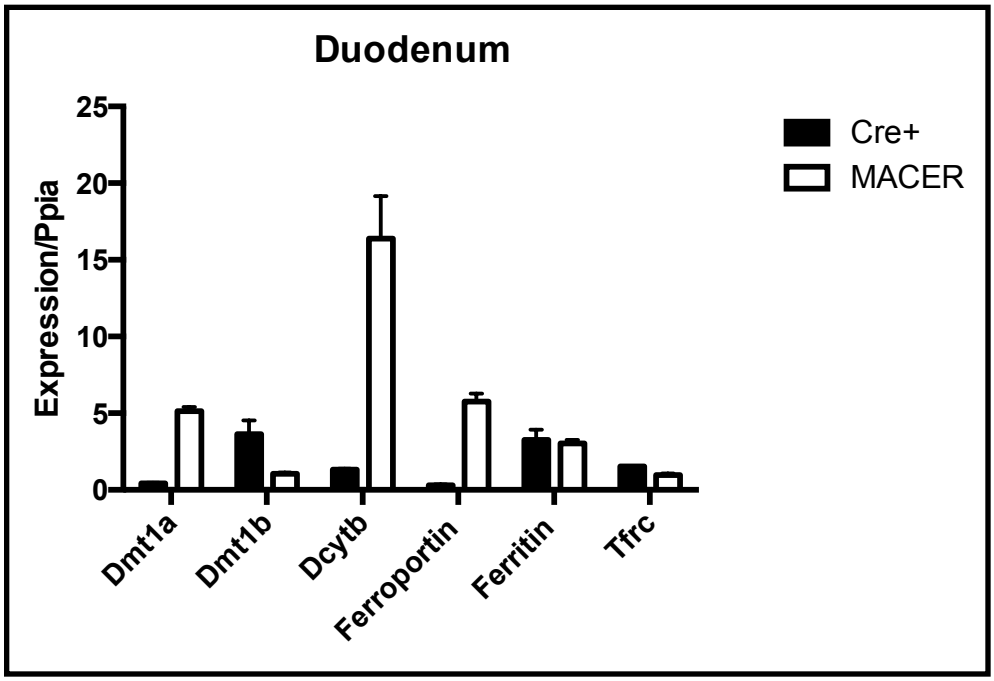

Figure 2.6: **Duodenal expression of iron transporters is altered in MACER mice.**
Basal serum iron compared to serum iron 2hrs after LPS-injection (500ng/Kg). (n=1). Error bars indicate mean +/- SEM. technical triplicates)

The Iron-retention phenotype in MACER mice increases with age and is gender-specific

Since peripheral iron accumulates with age, we decided to measure the iron levels in banked serum and liver samples from a cohort of 12 month old MACER female mice. We also had access to banked serum samples of age-matched male MACER mice and we tested serum iron and heme in these samples as well. We observed no difference in serum iron between male MACER and control mice, yet serum heme levels were strikingly lower in male MACER mice (2.7). This is in stark contrast to female MACER mice, which have no change in serum heme compared to control females. These results demonstrate that the regulation of iron and heme homeostasis is gender-specific and that ERα may play different roles in these processes in male and female mice. Further studies comparing macrophage iron homeostasis and heme metabolism in male and female mice are warranted.

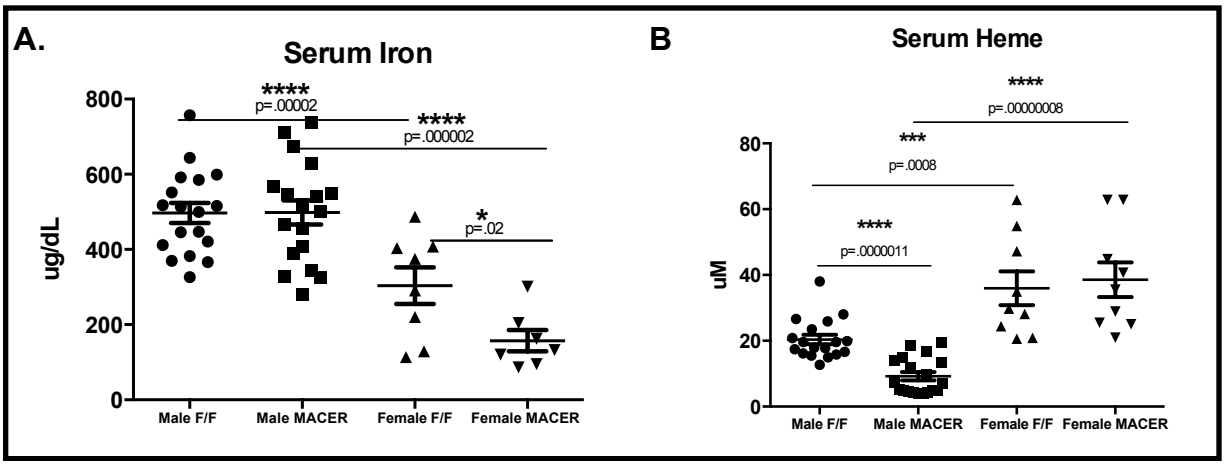

Figure 2.7: ERa deletion has gender-specific effects on iron and heme metabolism
A. Serum iron and B. serum heme measurements in a 12month old cohort of male and female control and MACER mice. Each dot represents one mouse. Error bars represent mean +/- SEM.

Discussion:

LysM cre deletion of *Esr1* had a subtle effect on the number of circulating monocytes and lymphocytes. We hypothesize that ERα deletion has a negative effect on the development and/ or behavior of these populations. Estrogen/ERα is known to influence many aspects of macrophage biology, including polarization and production of pro-inflammatory cytokines. Fewer circulating monocytes could dampen the hypoferremic response during infection, and we showed preliminary evidence to suggest this is true (2.5). The most profound phenotype from the CBC analysis was the 40% reduction of RBCs, hemoglobin, and hematocrit, the simultaneous reduction of which indicates severe anemia. Disruption of iron homeostasis in MACER mice was confirmed by the presence of low serum iron and the accumulation of iron in the spleen and liver. The major source of iron within macrophages is from the breakdown of hemoglobin during erythrophagocytosis. Macrophages break down hemoglobin into heme, which is further metabolized into free iron and bilirubin by hemoxygenase 1 (HMOX1). Heme levels were not different in MACER serum, spleen, or liver, indicating that defects in MACER macrophage iron recycling may be unrelated to the ability of MACER macrophages to catabolize heme from RBCs. However, MACER spleens had elevated expression of HMOX1, which suggests that MACER spleens are actively phagocytosing RBCs and generating free heme from hemoglobin.

Within the spleen, expression of ferrorporin and ferritin is restricted to red pulp macrophages and mRNA expression of these genes is significantly elevated in MACER whole spleen. The transcription and translation of ferroportin and ferritin is positively regulated by iron and the elevated expression of these genes in MACER spleen is consistent with the splenic iron accumulation in these mice. Under iron replete conditions, ferroportin and ferritin mRNA are unbound by IRP1/2 and freely translated to facilitate the storage and export of excess cytoplasmic iron, however ferroportin protein can be negatively regulated by hepcidin and we must confirm ferroportin protein expression. Since ferroportin is the only known iron exporter, we

believe that blockade of ferroportin protein expression in MACER mice is responsible for the observed peripheral iron accumulation.

To our surprise, the MACER phenotype is strikingly similar to the anemia and peripheral iron storage observed in Fpn1 KO mice and humans with ferroportin disease. At 2 months of age, Fn1KO mice also have a large increase in spleen and liver iron accumulation, along with a 22% decrease in serum iron and a 15% decrease in RBC count, hemoglobin, and hematocrit. The similarities between MACER and Fpn1KO phenotypes support our hypothesis that ERα is required for macrophage iron efflux via ferroportin. This seems to contradict our evidence that estrogen treatment represses ferroportin transcription, but ERα can also participate in hormone-independent gene regulation and therefore may differentially regulate ferroportin expression by both estrogen-dependent and estrogen-independent mechanisms. We hypothesize that estrogen/ERα negatively regulates ferroportin mRNA expression and that ERα also positively regulates ferroportin protein expression in a separate mechanism. As we pursued this study, another group confirmed that estrogen inhibits ferroportin transcription by inducing ERα to bind an estrogen response element on the ferroportin promoter. These in vitro studies were supported by the fact that ovariectomized (OVX) mice have elevated levels of ferroportin mRNA expression in the absence of estrogen signaling, however they did not examine ferroportin protein levels. They also examined the effects of estrogen deficiency on iron homeostasis in ovariectomized (OVX) mice and observed small, insignificant changes in serum iron, peripheral iron, RBC count, hemoglobin, and hematocrit that together resemble a very mild version of the MACER and Fpn1KO phenotype. This supports our hypothesis that ERα has an important, estrogen-independent role in the regulation of iron homeostasis that facilitates ferroportin protein expression.

We predict that ERα functions in one of the following models to regulate ferroportin expression and/or iron homeostasis. Two of our models directly implicate ferroportin protein function as the primary cause of the MACER phenotype (Fig 2.8, schematic). The other models illustrate

scenarios by which MACER anemia and iron accumulation is a secondary result of bone marrow defects or simply inflammation:

Model 1: Chronically elevated hepcidin levels block iron egress from spleen and liver macrophages:

The low serum iron levels in MACER mice should repress the expression of hepatic hepcidin to allow the flow of dietary iron from enterocytes and recycled iron from macrophages. While the contribution to circulating levels is unknown, macrophages are also capable of secreting hepcidin. It is possible that MACER red pulp macrophages and Kupffer cells secrete hepcidin, which then acts in an autocrine manner to block ferroportin and iron export from macrophages in the spleen and liver, respectively. In the proposed scenario, ferroportin protein function is blocked regardless of the status of liver hepcidin production and iron accumulates in macrophages after erythrophagocytosis. Multiple studies demonstrate the ability of ERα to bind estrogen response elements in the *Hamp1* promoter to negatively regulate hepcidin. This leads us to hypothesize that in the absence of ERα, hepcidin signaling may be elevated in MACER macrophages. Metal toxicity, oxidative stress and inflammation can all drive hepcidin expression in macrophages, and in further support of this model, we detected more ROS and superoxide production in shEsr1 RAW macrophages and elevated expression of pro-inflammatory cytokines in MACER bone marrow macrophages. We can measure hepcidin expression by MACER spleen and perform in vitro iron-loading studies with bone marrow macrophages to estimate the capability of MACER macrophages to secrete hepcidin in vivo.

Recent studies suggest that hepatocyte hepcidin secretion is also regulated by local iron concentration within the liver and that this regulation is dependent on mouse strain. The dependence of hepcidin mRNA expression on serum and liver iron was analyzed in multiple mouse strains. In the C57/Bl6 background, hepcidin expression is predominantly dependent on serum iron, whereas in the CD1 mouse strain, hepcidin is dependent on liver iron [8]. The

C57Bl/6 background of our mice suggests that hepcidin is predominantly regulated by serum iron, but it is important to consider the possibility that hepatic hepcidin expression can be dependent on both serum and liver iron concentration, which is higher in MACER mice. Therefore we must also measure serum and liver hepcidin levels to verify whether MACER hepatocytes secrete hepcidin.

Model 2: Altered homeostasis within the macrophage depletes the labile iron pool resulting in blockade of ferroportin protein translation by IRP1/2

Ferroportin translation is regulated by the labile iron pool and the ability of IRP1/IRP2 to bind the IRE on ferroportin transcripts. Under iron deficient conditions, IRP1/2 are free to bind the IRE in the ferroportin promoter and block translation. A scenario in which iron is sequestered in the endolysosomal system or mitochondria could deplete the labile iron pool and prevent translation of ferroportin despite cellular iron overload. This occurs in *Steap3* null mice, which lack the metalloreductase activity necessary to reduce Fe III to Fe II for transport into the cytoplasm [4]. As a result, iron accumulates in endolysosmes and lysosomes while the labile iron pool is depleted. The *Steap3* gene contains contains 15 potential estrogen response elements and we demonstrated that estrogen negatively regulates Steap3 expression in BMMs. We also observe elevated *Steap3* expression in MACER BMMs, which is further evidence that estrogen/ERα signaling negatively regulates *Steap3* expression. The expression levels of several other genes responsible for intracellular iron transport and metabolism also appear to be elevated in MACER BMMs and spleen. While our results suggest that intracellular iron transport and metabolism is functional in MACER mice, we cannot exclude the possibility that iron is sequestered within organelles in MACER mice. We can use scanning transmission X-ray microscopy can identify the presence of concentrated iron plaques within macrophages to help address this model [4].

Model 3: Peripheral iron accumulation in MACER mice is secondary to erythropoietic defects

Our data suggests that MACER mice have reduced circulating monocytes and thrombocytopenia in addition to severe anemia and iron overload. Iron deficiency anemia rarely causes a drop in platelet count and when these co-occur in humans the cause is typically a bone marrow disorder [5]. A particular subset of bone marrow macrophages is required for the development of erythrocytes in erythroblastic islands. These “nurse” macrophages tether to developing erythrocytes and are known to participate in erythrocyte enucleation and the transfer of iron and heme for hemoglobin synthesis [6]. The inability of resident MACER bone marrow macrophages to export vast quantities of iron for hemoglobin synthesis to erythroblastic islands can limit erythropoietic potential and cause anemia. This is a relatively new concept in macrophage biology and much work is needed to define the role of nurse macrophages in erythropoiesis. Flow cytometry analysis is routinely used to identify defects in the different stages of erythropoiesis utilizing antibodies against the late-stage erythroid marker Ter119 and the transferrin receptor (CD71). Inflammation also inhibits erythropoiesis, therefore the pro-inflammatory nature of MACER macrophages may locally influences erythropoiesis in the bone marrow. Spleen erythropoiesis occurs during acute anemia, where a population of resident immature erythroid de progenitors are expanded.

When we examined Esr1 correlations in bone, we observed correlations between Esr1 and regulators of iron metabolism (*Aco2, Frataxin)* as well as a correlation with *Vcam1* (r =.322), which is important for macrophage interactions with developing erythrocytes. *Vcam1* expression is already known to be regulated by estrogen/ERα [7], which raises the possibility that MACER macrophages are deficient for VCAM1 and have defective tethering to developing erythrocytes. This would negatively impact erythrocyte development cause anemia as observed in MACER mice. This model is supported by the fact that defective erythropoiesis can also cause iron overload by inhibiting hepcidin production while simultaneously increasing dietary iron absorption.

Model 4: The MACER phenotype is the result of anemia of inflammation

Iron sequestration in reticuloendothelial macrophages is a hallmark of inflammation because macrophages retain iron in response to inflammatory signals to limit bacterial growth in the blood. Chronic inflammatory disorders associated with elevated circulating IL-6 can induce hepatic hepcidin secretion resulting in anemia of inflammation. Circulating hepcidin blocks ferroportin and thus inhibits dietary iron acquisition and the release of iron from macrophages after erythrophagocytosis. The end result of chronic inflammation is peripheral iron accumulation and anemia. Estrogen/ERα signaling has known immunosuppressive functions in macrophages and may explain the elevated basal expression of inflammatory cytokines by MACER macrophages. We speculate that MACER macrophages constitutively secrete pro-inflammatory signals such as IL-6, which induce hepcidin expression and lead to the peripheral iron retention and anemia in MACER mice. We must measure serum IL-6 and hepcidin in MACER mice to address the validity of this model.

Here, for the first time, we demonstrate the necessity for macrophage ERα in the regulation of macrophage and systemic iron homeostasis. Further investigation into our proposed models will uncover novel mechanisms for iron regulation and result in a greater understanding of estrogen/ERα signaling in the maintenance of iron homeostasis, iron storage disorders, and pathologic iron accumulation after menopause. In general, estrogen/ERα signaling appears to prevent iron retention because estrogen treatment negatively regulates major iron-related genes and ERα deletion leads to the accumulation of iron. This finding is relevant for understanding the iron accumulation that occurs in women after menopause when circulating estrogen levels drop to the levels that are found in men. The gender differences in serum iron and heme levels observed in the banked serum samples prompts further studies to investigate gender dimorphic regulation of iron and heme metabolism.

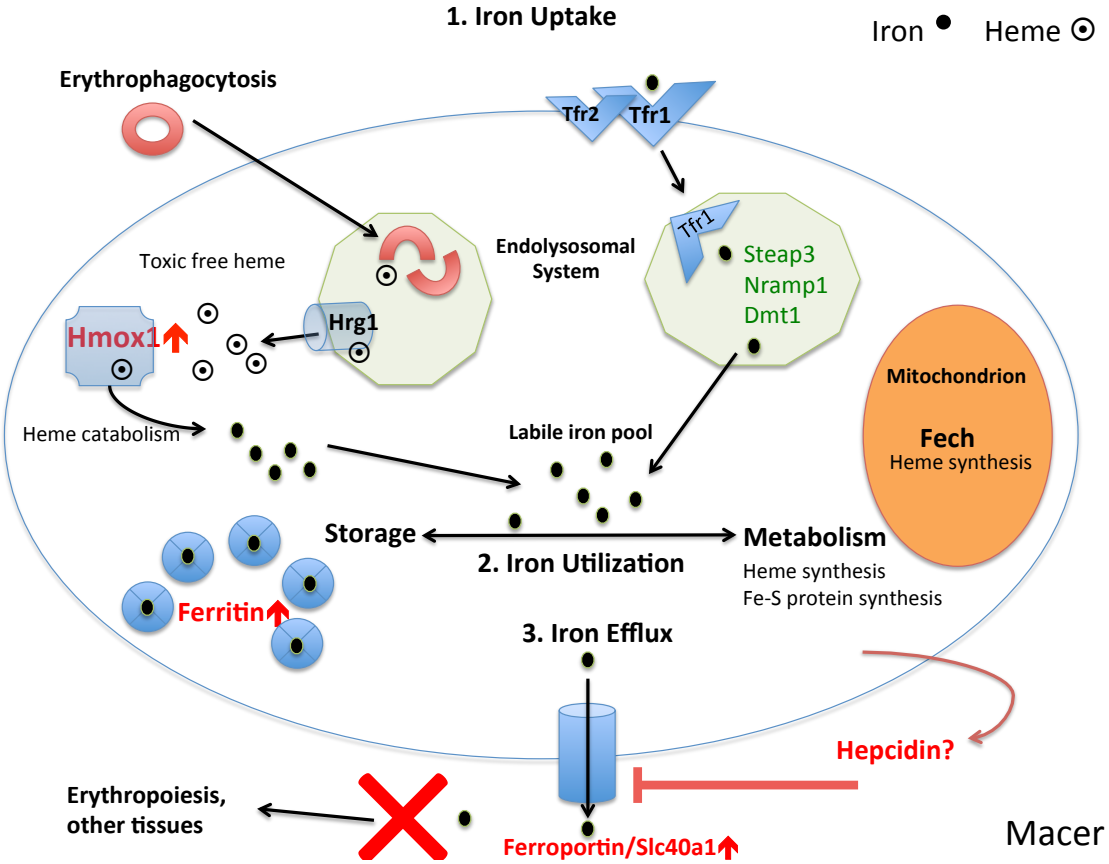

Figure 2.8: **Schematic depicting potential models for the anemia and iron retention in MACER mice**

Iron is taken up by transferrin or via erythrophagocytosis. Heme iron is exported into the cytosol by HRG1 where it is catabolized by HMOX1 into iron, bilirubin, and CO2. Iron in the labile iron pool is either shuttled to mitochondria for protein and heme synthesis, stored as ferritin, or exported from the cell via ferroportin. We hypothesize that ferroportin blockade by hepcidin or through translation inhibition inhibits iron efflux from MACER macrophages and limits iron for erythropoiesis, resulting in anemia.

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CHAPTER THREE: UNCOUPLING EFFECTS OF GENDER AND SEX HORMONES ON MACROPHAGE BIOLOGY AND THE REGULATION OF IRON HOMEOSTASIS

Introduction

Sexually dimorphic immune function contributes to gender differences in biology and disease susceptibility

Sexually dimorphic gene expression, epigenetics, microbiota, and sex chromosomes all contribute to differences in immune cell behavior and function [1-3]. The X chromosome contains 5% of the genome and encodes 16 immune receptors and receptor-related proteins, 15 immune response-related proteins, and 28 transcriptional and translational effectors of the immune system [1]. One of two X chromosomes are randomly inactivated in female cells, creating a mosaic expression of either the maternal or paternal X chromosome during female development. This protects females from X linked diseases and ensures females have a more diverse repertoire of proteins which can influence physiological processes. This may partially explain why men are more susceptible to a host of different bacterial, viral, and fungal infections and are much more likely to die from sepsis compared to women [4]. On the other hand, there appears to be a biological tradeoff where the sexual differences in female immunity confer protection against certain infections at the cost of heightened risk for autoimmunity. Women are disproportionately affected by autoimmune diseases including systemic lupus erythematous (9:1), multiple sclerosis (2:1), and rheumatoid arthritis (2.5:1) [5]. Women also mount a more robust immune response to vaccines and infections than men and are partially refractory to the effects of anti-inflammatory drugs such as aspirin and statins [6-7].

A large body of evidence supports a role for estrogen/ERα signaling in innate and adaptive immune responses. Elucidating the signaling mechanisms has proved challenging, because estrogen/ERα can modulate both pro- and anti-inflammatory immune responses. The strongest

evidence supports a role for estrogen/ERα in the induction of type I interferons [8], the induction of TLR-mediated pro-inflammatory cytokine production [9], and in limiting NF-kB-dependent inflammatory responses [10]. The amount of estrogen signaling also appears to be critical in determining whether a pro-inflammatory or anti-inflammatory response is elicited: low levels of estrogen appear to mediate pro-inflammatory responses, while high concentrations promote anti-inflammatory responses. This is supported by the inverse relationship between circulating estrogen and pro-inflammatory cytokines [11]. High doses of estrogen treatment can also improve mortality by reducing the levels of pro-inflammatory cytokines [12].

ERα may have a particularly important role for regulating sexually dimorphic macrophage function, because unlike lymphocytes, ERα is differentially expressed in human monocytes. Male monocytes express more ERα than monocytes from pre-menopausal women. Instead, ERα expression by male monocytes is similar to those isolated from post-menopausal women. This is thought to occur because ERα expression negatively correlates with circulating estrogen levels. Furthermore, there are number of differences in gene expression in circulating monocytes between pre- and postmenopausal women [13].

Iron metabolism by macrophages is directly influenced by inflammatory signals in general and by TLR signaling in particular, therefore it is highly likely that estrogen/ERα signaling contributes to both macrophage and systemic iron homeostasis. It is entirely possible that male and female macrophages metabolize iron differently based on our preliminary findings. The fact ERα is a female sex hormone receptor increases the likelihood that ERα differentially regulates iron metabolism in male and female macrophages.

Methods

Animals: Age-matched wild type male and female mice were obtained from JAX. Mice were maintained in pathogen-free facilities at the University of California, Los Angeles (UCLA). All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Animal Research Committee of UCLA. Results are consistent with two different experiments consisting of n=3/4 animals per gender.

Serum Iron Analysis: Mouse blood was collected and centrifuged for 5min at 4500rpm. Supernatant was removed, centrifuged again, and the new supernatant was put in a fresh tube and frozen at -80°C for storage before iron analysis. In a 96-well assay plate, 10uL serum from each mouse was plated in three or more replicate wells. Serum iron content was determined with the colorimetric Iron-SL Kit (Sekisui) by adding 150uL acid dissociating buffer and 40uL color reagent to each well. Plates were read at 595nm after a 5 minute incubation and iron concentration was determined using a multi-point standard curve.

Tissue Iron Analysis: A small piece of flash frozen liver from each mouse was weighed in a pre-tared microcentrifuge tube and homogenized in 1125uL protein precipitation buffer (.53N HCL and 5.3% trichloroacetic acid in HPLC water). Samples were boiled for 1 hour and then centrifuged for 10 minutes at maximum speed. The supernatant was recovered and stored at -80°C for iron analysis. 30uL of the tissue supernatant was used in each well and the iron assay was performed the same as with serum

Serum and Tissue Heme Analysis: Serum and tissue samples were prepared the same as for iron analysis. Sample heme concentration was determined using the colorimetric Quantichrome Heme Assay Kit (Bioassay Systems). 25uL of either serum or tissue supernatant were assayed

in triplicate after a 5min incubation in 200uL heme kit reagent. Absorbance was read at 400nm and heme concentration was calculated with a multi-point standard curve.

Quantitative PCR: RNA was extracted using TriZol reagent and RNeasy columns (Qiagen). cDNA synthesis was performed using 1µg of RNA with SuperScript II reverse transcriptase (Invitrogen). Gene expression analysis was performed on a BioRad MyiQ real time detection system using iQ SYBER Green Supermix (BioRad). Genes are expressed as relative mRNA level compared with the housekeeping gene PPIA. Primer pairs were used based on published sequences (below). When indicated, datasets from separate cohorts/experiments were combined by normalizing the expression of control animals to 1.

Cell Culture: Both femurs and tibias from a wild type C57Bl/6 female mouse were flushed with MACS buffer (PBS + 2% FBS and 1mM EDTA). After RBC lysis, bone marrow cells were counted on a hemacytometer and plated in L929 medium (DMEM+ 10% FBS + 30% L929 conditioned media) at a density of 10^6 cells/mL in non-TC treated 20cm plates. Media was refreshed on day 3 of differentiation and day 7 mature macrophages were plated in triplicate at the same density into 6-well plates for experiments. Cells were treated with 10nM Estrogen for 24 hours before lysing cells in TriZol reagent for RNA isolation.

Immunoblot Analysis:

Lysates were obtained from day 7 bone marrow macrophages homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Protein concentration was determined with a BCA protein assay kit (Pierce) and 20ug of protein was loaded per well for immunoblots.

Flow cytometry:

Cell surface markers: Single-cell suspensions of day 7 bone marrow macrophages were incubated with Fc block before staining with fluorescently labeled primary antibodies for 30 min

at 4 °C. F4/80-allophycocyanin (APC), MHC-class II-PE, CD11c-PE-CY7, and CD11c-Percp-Cy5.5 antibodies were used. Cells were stained for 30 min at 4 °C before, washed 3x's in PBS, and resuspended in 400uL FACS buffer for flow cytometry. For additional assays, basal or LPS-treated day 8 bone marrow macrophages cells were stained with 10 mM DCFDA, 100nM Mitotracker Green, 75nM Lysotracker Red, or 5uM Mitosox (all from LifeTechnologies) for 20 minutes at 37°C and washed 3x's with PBS before resuspension in 400uL FACS buffer. Cells pre-treated with 1uM antimycin A for 15 minutes served as positive controls. Flow cytometry was performed on a LSRII and data was analyzed using FlowJo.

Phenotyping: Freshly isolated bone marrow or spleen cells were incubated in 0.5ug Fc Block (BD Biosciences) in PBS/0.5% BSA for 10-minutes at room temperature. Cells were stained with fluorescently-labeled primary antibodies on ice in the dark for 30 minutes and washed three times in PBS/0.5% BSA before data acquisition on a LSRII. A comprehensive list of surface markers for flow cytometry experiments includes: CD3 clone 145-2C11 PE (1:300 eBioscience), CD3 clone 145-2C11 APC-Cy7 (1:200 eBioscience), CD4 clone RM4-5 PE (1:300, Invitrogen), CD4 clone RM4-5 PE (1:200, Invitrogen), CD8 clone 53-6.7 Pb (1:200, Pharmigen), CD8 clone 53-6.7 PE (1:300, Pharmigen), Cd11b clone m1/70 Pe-Cy7 (1:300, eBioscience), CD11b clone m1/70 PE (1:300, eBioscience), Cd11c clone n418 PerCp-Cy5.5 (1:200, eBioscience), CD34 clone RAM34 AF700 (1:200, eBioscience), Cd16/34 clone 93 (1:300, Bioglegend), CD86 clone B7-2 Fitc (1:200, eBiosceince), CD117/cKit clone 2B8 Fitc (1:200, eBioscience), CD45/B220 clone RA3-6B2 PE (1:300 eBioscience), CD45/B220 clone RA3-6B2 AF610 (1:200, eBioscience), CD19 ID3 APC-Cy7 (1:200, eBioscience), CD19 ID3 Pacific Blue (51:200, eBioscience), Sca-1 clone D7 PerCP (1:200, eBioscience), Ter119/Ly76 clone TER119 PE (1:300, eBioscience), F4/80 clone BM8 APC (1:200, eBioscience), Gr-1 PE clone RB6-8C5 (1:1000, eBioscience), Ly6c clone HK1.4 Fitc (1:200, Biolegend), Ly6g clone 1A8 APC (1:200, Biolegend) , MHCII clone M5/114.15.2 APC (1:1000, Biolegend), MHCII clone M5/114.15.2 AF700 (1:1000, Biolegend).

Results

Estradiol treatment alters the expression of iron-related genes in male and female macrophages

We previously observed that iron-related genes contain multiple estrogen response elements (EREs) and respond to estradiol treatment in female bone marrow macrophages (BMMs). Female *Esr1* expression in BMMs is only 40% of that of male BMMs (3.2a). This is consistent with studies conducted on *Esr1* expression by male and female circulating monocytes. Of note, it appears that gender differences in *Esr1* expression persist in culture and that hormone-independent regulation of ERα expression occurs in both male and female cells. We next exposed BMMs to estrogen to learn how iron metabolism genes respond to estrogen in both genders. While male macrophages have higher basal expression of *Dmt1, Dmt1b, Nramp1,* and *Steap3* mRNA, estrogen-dependent changes in gene expression are highly similar between genders (3.1a-c). Estrogen also induces the expression of the rate limiting enzyme in heme synthesis, FECH, and negatively regulates HMOX1, which catabolizes heme (3.1b). It appears that estrogen can regulate macrophage iron metabolism regardless of gender and that the general effect of estrogen is to negatively regulate iron reductases, intracellular transporters and iron export, and to positively regulate heme synthesis. Many genes involved in macrophage iron homeostasis respond to inflammatory stimuli as part of the hypoferremic response to sequester iron from the blood during infection. We next exposed male and female BMMs to LPS to determine if there are gender differences in the regulation of iron metabolism in response TLR4 activation (3.2a). LPS treatment greatly diminishes *Esr1* expression in males and has no effect on female *Esr1* expression. LPS-induced changes in iron metabolism gene expression were gender-specific for many of the genes we examined. NRAMP1 and DMT1a/b isoforms control the intracellular transport of iron in phagosomal membranes and throughout the endolysosomal system, respectively and were significantly changed in females, but not males. It is possible that

females are more responsive to LPS in terms of altering cellular iron metabolism. LPS has only subtle effects on the expression of heme-metabolism genes, with the exception of *Hmox1,* which is induced 5-fold in male macrophages and 2-fold in female macrophages. Expression of pro-inflammatory cytokines was similar between genders (3.2b).

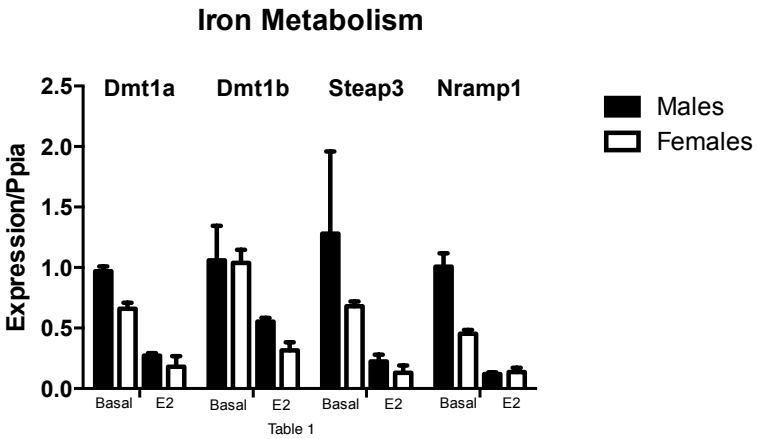


Table 1

	Dmt1a	Dmt1b	Steap3	Nramp1
Male p value	0.009	0.08	0.17	0.001
Female p value	0.009	0.005	0.002	0.009
# ERE's	8	8	15	3
Ct	27	25	25	23

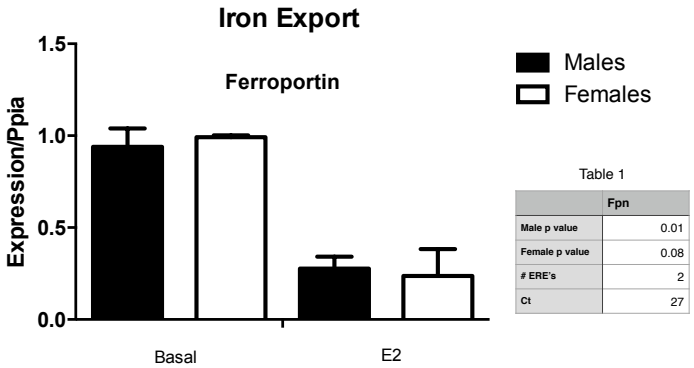


Table 1

	Fpn
Male p value	0.01
Female p value	0.08
# ERE's	2
Ct	27

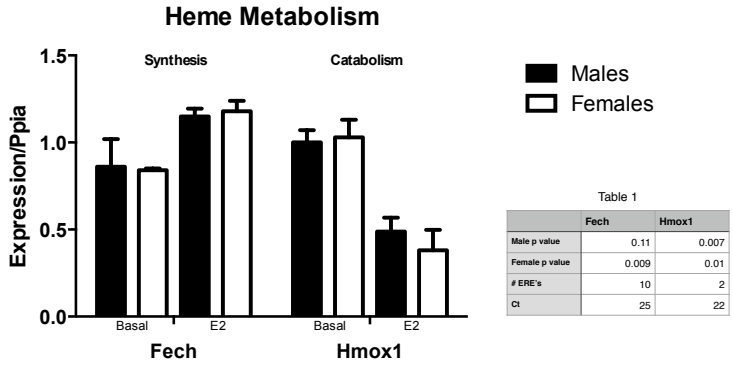


Table 1

	Fech	Hmox1
Male p value	0.11	0.007
Female p value	0.009	0.01
# ERE's	10	2
Ct	25	22

Figure 3.1: Male and female macrophages alter the expression of iron metabolism genes in response to estrogen
Gene expression analysis of A. intracellular iron metabolism genes. B. Iron export, and C. heme metabolism. Statistics represent mean +/- SEM (n=3).

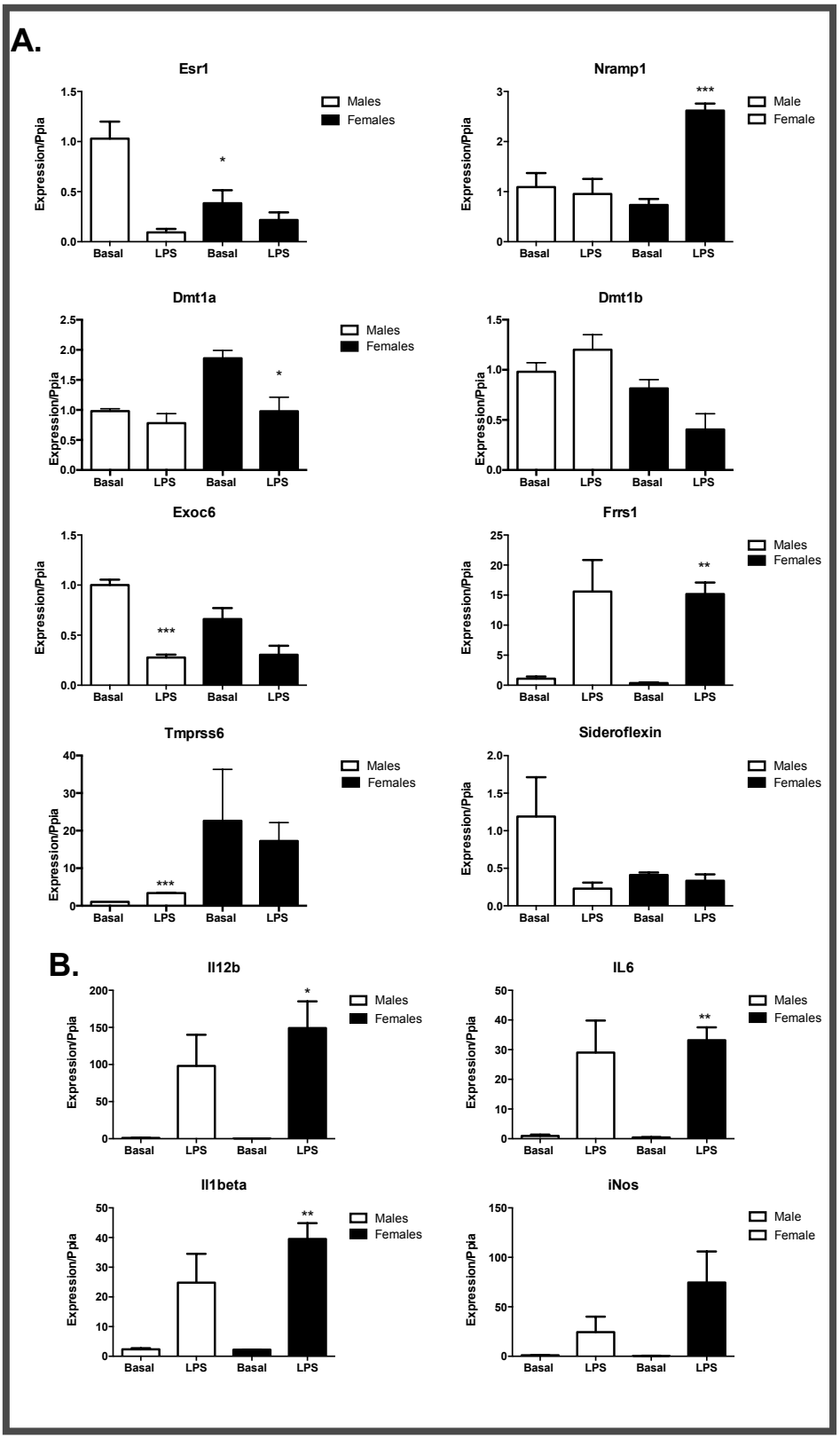


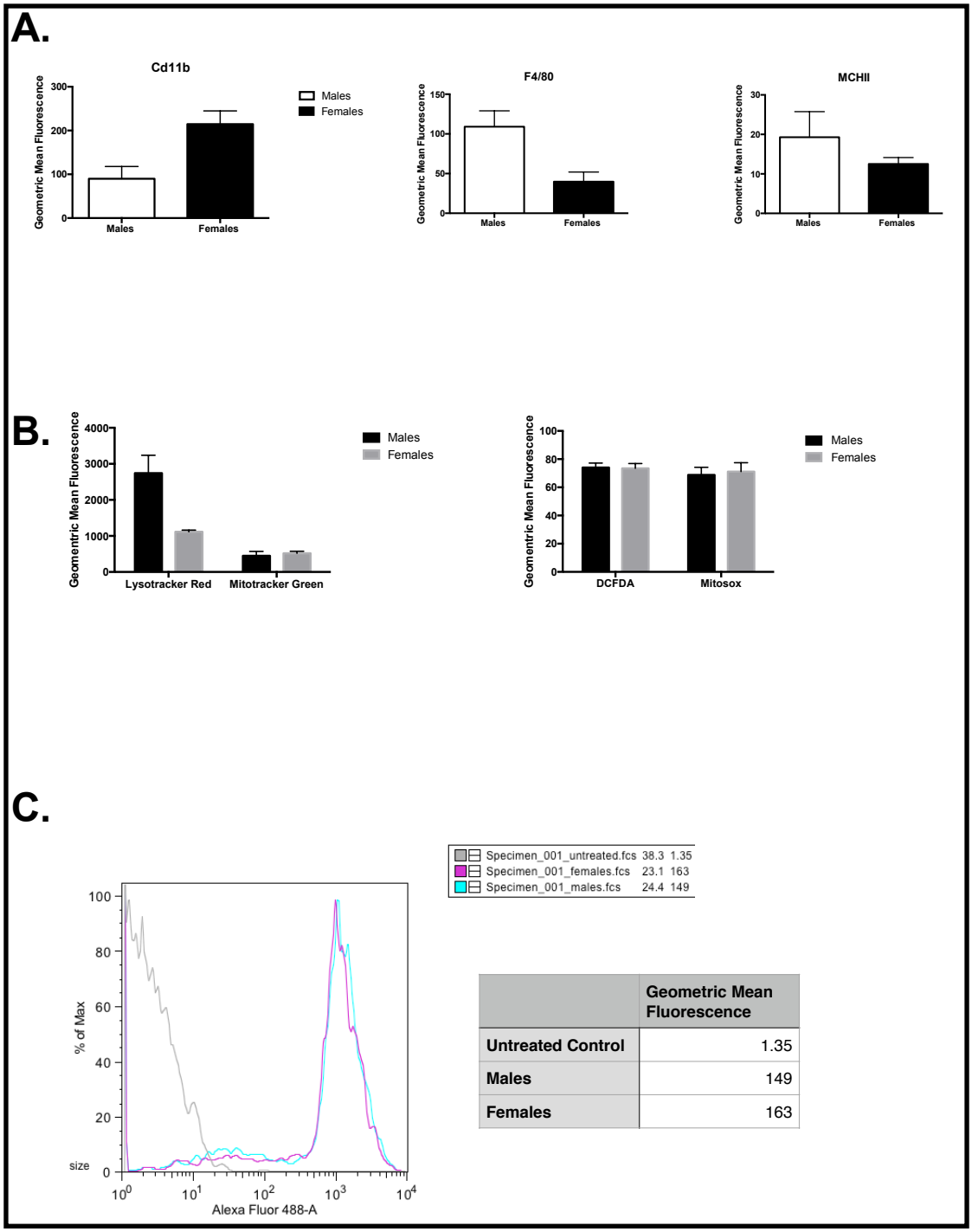
Figure 3.2: Differential response of iron genes to LPS in male and female macrophages. Expression of A. Iron metabolism genes and B. pro-inflammatory cytokines in bone marrow macrophages. Statistics represent mean +/- SEM (n=3 wells).

Macrophage markers have gender-specific cell surface expression:

Male and female macrophages express different levels of macrophage markers such as CD11b (higher), F4/80 (lower), and MCHII (lower) (3.3a). Cd11b is an integrin family member expressed on leukocytes to mediate the inflammatory process via regulating adhesion and migration. Higher Cd11b expression by female macrophages could help explain why these cells resolve infections faster than male macrophages. Gender differences in the expression of macrophage markers should be considered because they likely influence the detection of macrophages across multiple platforms which utilize antibodies against macrophage surface markers.

Female macrophages have less lysosome content:

The majority of cellular iron is metabolized in the endolysosomal system and mitochondria. To investigate whether male and female macrophages have innate differences in the quality and quantity of the machinery required for iron metabolism, we utilized a panel of flow cytometry probes: Mitotracker (mitochondria content), Lysotracker (lysosome content), Mitosox (mitochondrial derived superoxide), DCFDA (total cellular reactive oxygen species). There is no difference in the staining for mitochondria, superoxide production, and total reactive oxygen species production in male and female BMMs (3.3b). Surprisingly, the mean fluorescence for lystoracker is more than 50% lower in female cells. We observed the same result when we measured lysotracker fluorescence in adherent cells in a plate reader-based assay. Immunoblots for the lysosome-associated protein, LAMP1, also show lower expression by female macrophages and add to the evidence suggesting reduced lysosome content in female BMMs (3.5a).



	Geometric Mean Fluorescence
Untreated Control	1.35
Males	149
Females	163

Figure 3.4: Flow cytometry phenotyping of male and female macrophages
A.Relative expression of macrophage markers. Geometric mean fluorescence, Error bars indicate mean +/- SEM, n=3.
B.Geometric mean fluorescence of stains for mitochondria, lysosome, and ROS markers Geometric mean fluorescence, Error bars indicate mean +/- SEM, n=3.
C. Geometric mean fluorescence of Fitc-labeled E.coli phagocytose by male and female macrophages (n=1)

Female bone marrow macrophages express fewer ER stress markers:

There is substantial evidence in the literature that LPS and cellular stressors such as excess ROS and ER stress can all induce hepcidin and regulate cellular iron metabolism. ER stress has been shown to increases hepcidin expression via CREBH and STAT3 [14]. C/EBPa can both positively and negatively regulate hepcidin by binding the hepcidin promoter. When we examined whether there are intrinsic differences in the expression of ER stress markers, we saw that males express more pSTAT3, pEIF2alpha, pPERK, and XBP1s than females (3.5). These differences exist despite identical culture conditions and the absence of phenol and exogenous hormones, implying that there are intrinsic gender differences in macrophages that contribute to ER stress.

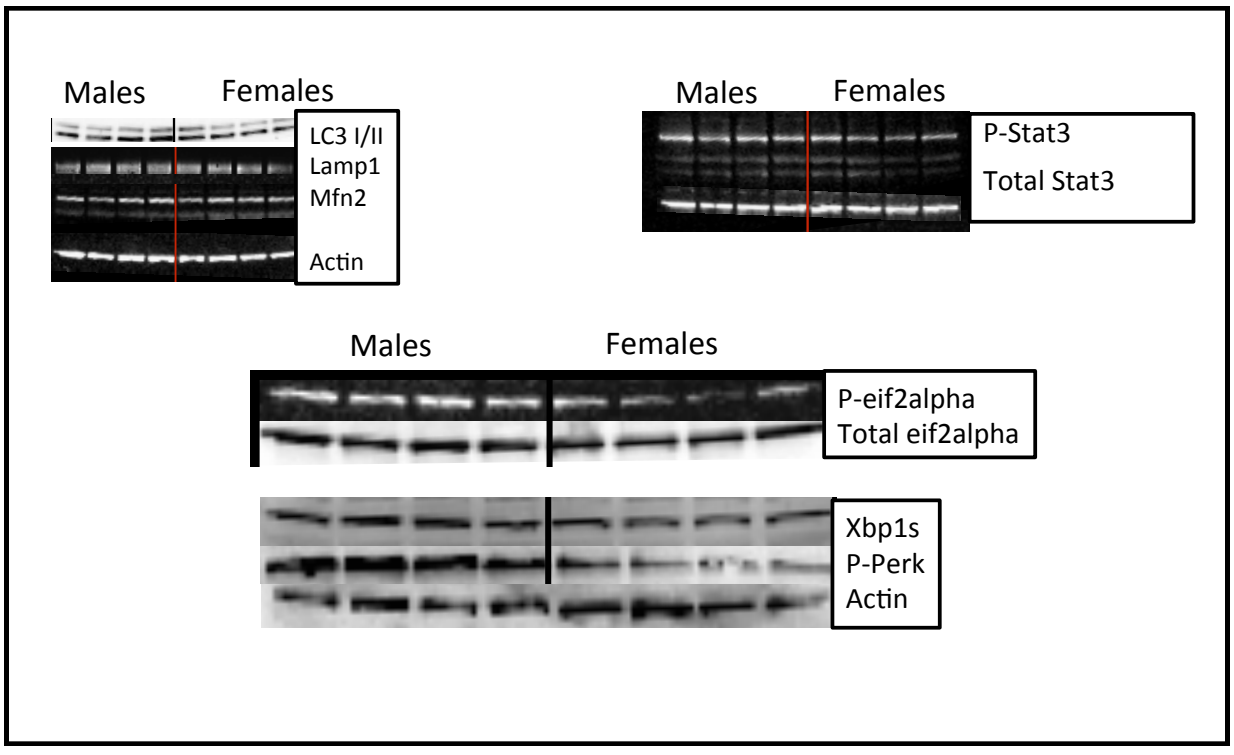


Figure 3.5: Sexually dimorphic expression of lysosomal and ER-stress markers in male and female macrophages
Immunoblots of ER stress markers in day 8 bone marrow macrophages (n=4)

Discussion

In this study, we investigated gender-specific regulation of iron metabolism and differences in macrophage metabolic parameters that may contribute to sexually dimorphic immunity. Our gene expression analysis revealed male and female bone marrow macrophages (BMMs) respond similarly to exogenous estrogen treatment in terms of iron-regulatory gene expression. Interestingly, male BMMs have much higher basal *Esr1* expression than females and retain this expression pattern in culture, suggesting that sex-specific *Esr1* expression can be independent of sex hormones. This is in contrast to the notion that in humans, lower *Esr1* expression by female monocyte is a solely the result of higher circulating estrogens. There were differences in LPS-dependent changes in several iron metabolism genes, particularly for the gene encoding intracellular iron transporter, *Nramp1.* NRAMP1 protein localizes to phagosomal membranes and transfers iron from within the phagosome to the cytosol to sequester iron from bacteria. Pathogens have multiple methods to scavenge iron from their environment, including hosts such as macrophages, and microbial pathogenesis is tightly linked to the ability of microbes to acquire iron. Therefore, macrophages must be efficient at sequestering iron from engulfed pathogens and NRAMP1 is essential to this process. Failure of male macrophages to induce *Nramp1* expression in response to inflammatory signals may influence the ability of male macrophages to control the growth of intracellular pathogens after phagocytosis. It is possible that differences in phagosomal iron sequestration contribute to the higher risk of sepsis in males. Investigating gene expression changes after LPS stimulation at additional time points will reveal if there are gender differences in the kinetics of iron metabolism under inflammatory conditions.

Of the parameters we examined, the most striking difference between male and female macrophages was lysosomal content. The reduced lysosomal content we observed in female macrophages is likely to influence iron metabolism because of the critical role for lysosomes in

iron recycling. A reduced capacity to recycle iron could also render females more susceptible to toxic overload. Since women are more likely to accumulate peripheral iron in aging, one can speculate that gender differences in cellular machinery responsible for iron recycling contribute to this observation.

Endoplasmic reticulum (ER) stress, which activates STAT3 signaling, has recently been added to the list of hepcidin regulators. CREBH (cyclic AMP response element-binding protein H) is a transcription factor activated by ER stress which has been shown to bind the hepcidin promoter to induce expression. On the other hand, protein levels of CHOP (C/EBP homologous protein) protein levels also rise during ER stress and this leads to a decrease of C/EBPa, which may lead to reduced hepcidin transcription [14]. While it is unclear why male macrophages have more ER stress signals or if these signals influence iron metabolism, this finding may have important implications in underlying sexual differences in macrophage function and behavior because ER stress modulates M2 polarization and TLR signaling.

Gender differences in iron metabolism have direct implications in the control of macrophage polarization. M1 macrophages repress ferroportin expression and induce ferritin expression as part of the hypoferremic response of infection to reduce serum iron levels. M2 macrophages have the opposite expression pattern and favor iron release. It is hypothesized that the intracellular iron content of M2 macrophages is low and thus limits the formation of iron-containing enzymes necessary for pro-inflammatory response. It is therefore possible that iron plays a key role in dictating macrophage polarization by limiting the inflammatory response in M2 macrophages and altering the polarization state of macrophages as they enter iron-rich tissues. Future studies need to examine iron homeostasis by male and female macrophages under different polarization conditions to understand if regulation of macrophage polarization by iron (or vis versa) contributes to sexual dimorphic immune responses.

Despite the body of evidence for sexual dimorphism in most biological subfields, there is a bias towards male research subjects at the levels of clinical trials, mouse models, and cell lines. With the exception of reproduction and endocrinology studies, females have been historically excluded from research due to concerns about reproducibility and the need to time experiments for cycling rodents. In the past decade, clinical observations and immunological studies have revealed striking differences between male and female immune responses and this has prompted researchers to include female mice in their studies and to examine gender-specific immune regulation. The intrinsic differences in macrophage iron metabolism, lysosome content and ER stress identified by this study support the case for gender equality in research and have broad implications for future studies on gender differences in macrophage biology.

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