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The Use of Mouse Models for Understanding the Pathogenesis of Anemia of Inflammation

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The Use of Mouse Models

for Understanding the Pathogenesis of Anemia of Inflammation

A dissertation submitted in partial satisfaction of the

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in Cellular and Molecular Pathology

by

Airie Kim

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ABSTRACT OF THE DISSERTATION

The Use of Mouse Models

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by

Airie Kim

Doctor of Philosophy in Cellular and Molecular Pathology

University of California, Los Angeles, 2013

Professor Tomas Ganz, Chair

Iron regulation during times of inflammation is largely driven by the hepcidin-ferroportin axis. Hepcidin expression increases with cytokine activity, particularly IL-6, and causes the internalization and degradation of the transmembrane cellular iron exporter, ferroportin. The impairment of iron efflux into circulation decreases the available supply for erythropoiesis, leading to iron restriction and anemia. Inflammation and increased hepcidin work together to produce the characteristic phenotype of anemia of inflammation (AI): a mild to moderate normocytic anemia with iron restriction, intact iron stores, a shortened erythrocyte lifespan, and depressed erythropoiesis. Evolutionarily, this mechanism limited the availability of iron to microbes during times of infection. However, AI also leads to poor quality of life with increased morbidity and mortality in patients with primary inflammatory conditions.

In this work, we address the need for mouse models of inflammatory anemias. The characterization and manipulation of these models enabled us to elucidate the pathogenesis of AI. Chapter 2 details a mouse model of aging using both wild-type (WT) and genetically manipulated mice. We showed that aging WT mice had increased inflammatory parameters, with hematologic and iron parameters that were consistent with AI. Repeating the model in IL-6 -/- and hepcidin -/- mice demonstrated a partial contribution of IL-6 and hepcidin to the anemia of aging.

Chapter 3 describes four different mouse models of anemia of cancer (AC), with varying degrees of chronicity. Our models displayed a spectrum of anemia severity, and manifested both iron-deficiency and inflammatory anemias. The phenotypes of the different mouse models truly illustrated the heterogeneity of AC cases in human patients. Repeating the experiments in hepcidin -/- mice showed no obvious contribution of hepcidin to AC in our models.

In chapter 4, we extensively characterized a mouse model of acute inflammation using *Brucella abortus*. We described a model with severe inflammatory anemia, early hepcidin increase, iron-restricted erythropoiesis despite iron accumulation in the liver, shortened erythrocyte lifespan, and suppressed erythropoiesis. With hepcidin ablation, we showed that there was a significant but partial contribution of hepcidin to the development of AI in the *Brucella abortus* mouse model.

In conclusion, we have characterized mouse models of anemia of aging, anemia of cancer, and anemia of sepsis, in order to elucidate the role of hepcidin and provide a research tool for potential future therapeutics.

The dissertation of Airie Kim is approved.

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ACKNOWLEDGMENTS

Chapter 2 is a reprint of: **"**Investigation of the role of interleukin-6 and hepcidin antimicrobial peptide in the development of anemia with age." Authors: McCranor BJ, Langdon JM, Prince OD, Femnou LK, Berger AE, Cheadle C, Civin CI, Kim A, Rivera S, Ganz T, Vaulont S, Xue Q, Walston JD, Roy CN. Originally published in Haematologica, 2013 Oct;98(10):1633-40. DOI: 10.3324/haematol.2013.087114. Reprinted here with permission from the Ferrata Storti Foundation.

Chapter 3 is a version of a manuscript in preparation: "Four mouse models illustrate different mechanisms of anemia of cancer." Authors: Airie Kim, Seth Rivera, Dana Shprung, Donald Limbrick, Victoria Gabayan, Elizabeta Nemeth, Tomas Ganz.

Chapter 4 is a version of a manuscript that has been submitted to Blood in 2013, titled "A mouse model of anemia of inflammation: complex pathogenesis with partial dependence on hepcidin." Authors: Airie Kim, Eileen Fung, Sona G. Parikh, Erika V. Valore, Victoria Gabayan, Elizabeta Nemeth, Tomas Ganz.

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Kim A, Fung E, Parikh S, Gabayan V, Nemeth E, Ganz T. *Detailed characterization of the mouse model of anemia of inflammation caused by heat-killed Brucella abortus reveals multiple causes of anemia and partial dependence on hepcidin.* Podium presentation at International BioIron Society conference "Best of Posters" session, 2013 April. London, England.

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Chapter 1

INTRODUCTION

IRON PHYSIOLOGY AND HOMEOSTASIS

Iron is an essential nutrient for nearly all organisms. In mammals, this element is used as a component of heme, including in hemoglobin and myoglobin, and in iron-containing centers in important redox enzymes [1]. The involvement of iron in vital cell activities, including cell proliferation [2] and DNA repair [3] requires adequate amounts of this element for cell health and proliferation. Conversely, iron overload can lead to cellular toxicity via iron-generated oxyradicals and peroxidation of lipid membranes [4]. In order to maintain the appropriate balance, organisms have developed complicated cellular and systemic mechanisms for iron homeostasis.

While this element is abundant on Earth, it is insoluble in aqueous solutions, so organisms have developed elaborate mechanisms to obtain and distribute iron in multicellular organisms [5]. Dietary heme and non-heme iron are absorbed in the proximal small intestine, where iron travels across the apical and basolateral membranes of the enterocytes in order to access the circulation. Heme iron is transported intact via a heme carrier protein [6]. Figure 1-1 summarizes the mechanism of nonheme absorption through the enterocyte. Nonheme iron is reduced by ferric reductases to Fe^{2+} [7] before being transported across the apical cellular membrane by divalent metal transporter 1 (DMT1) [8]. The iron is then transported across the basolateral membrane via ferroportin, the only known cellular iron exporter. This export of iron requires a ferroxidase activity, which is supplied by ceruloplasmin and hephaestin, a ceruloplasmin homolog [9]. This oxidation of Fe^{2+} to Fe^{3+} allows iron to circulate bound to plasma proteins. Absorbed iron is then bound to transferrin (Tf), which binds nearly all plasma iron.

Once in circulation, Tf-bound iron is taken up into cells through a TfR1-mediated process [10]. Iron moves into endosomes, where it is separated from the Tf before being transported back to the cytoplasm where it is largely stored in ferritin, a protein cage that can achieve impressive concentrations of stored iron [11]. The production of many of these proteins that function in iron uptake and storage is regulated by the iron levels of the cells themselves. In states of low intracellular iron, iron regulatory proteins 1 and 2 bind to iron regulatory elements in the mRNAs of iron-regulated proteins [12].

An average adult human has about 3-4 g of total body iron [13]. Iron losses account for only 1-2 mg/day in the form of epithelial sloughing and bleeding, and there are no known regulated mechanisms for iron excretion. About two-thirds of the iron in the body exists in heme in red blood cells, and about 20 mg of iron is lost each day from the death of erythrocytes. Because dietary iron absorption only provides sufficient iron to compensate for the daily losses (1-2 mg), 20 mg of iron/day must be obtained from the recycling of iron from old red blood cells. The cells are phagocytized by macrophages, heme is freed from hemoglobin, and then is degraded by heme oxygenase to release iron for reuse. Thus, proper iron homeostasis is maintained by adequate iron intake and absorption, minimal blood loss, and the recycling of iron from dying erythrocytes.

HEPCIDIN-FERROPORTIN AXIS IN IRON REGULATION

Ferroportin is a multi-spanning transmembrane transporter that functions as the only known pathway for cellular iron export, and is abundant on duodenal enterocytes, macrophages, hepatocytes, and placental trophoblasts [14]. The primary known regulatory of ferroportin is hepcidin, a 25-amino acid peptide hormone that is produced by hepatocytes and excreted in the

urine [15]. Because of its structural homology to antimicrobial peptides, hepcidin was initially believed to have antimicrobial properties. However, its principal described role is as the primary regulator of systemic iron homeostasis. Figure 1-2 provides a schematic of the hepcidin-ferroportin axis regulating systemic iron homeostasis.

Inactivation of the hepcidin gene in in mice causes a phenotype of severe iron overload [16, 17], and mutations in the hepcidin gene are associated with juvenile hemochromatosis, a particularly severe form of genetic iron overload [18]. Conversely, transgenic hepcidin mice with hepcidin overexpression develop the opposite phenotype of iron-deficiency anemia [19]. Similarly, human subjects with type 1a glycogen storage disease and liver tumors develop hepcidin overproduction and iron-deficiency anemia [20].

Hepcidin acts by binding to ferroportin and causing its internalization and degradation within lysosomes [21] (Figure 1-3). In one study, injecting a single dose of synthetic hepcidin into mice caused a rapid and dramatic drop in serum iron [22]. The circulating hepcidin binds to this sole known cellular iron exporter on macrophages, hepatocytes, and enterocytes, and disrupts the export of iron into circulation. Thus, the flow of iron from gut absorption, recycling of senescent erythrocytes, and cellular iron stores is impaired in times of increased hepcidin.

HEPCIDIN REGULATION

Hepcidin production is primarily regulated by iron concentrations, erythropoiesis, and inflammation (Figure 1-4).

Hepcidin regulation by iron and erythropoiesis

Hepcidin production is strongly regulated by iron status. TfR1 and TfR2 appear to be the two transferrin receptors that interact with the membrane protein HFE in order to sense holotransferrin levels [23, 24]. In times of increased iron, a FeTf/HFE/TfR2 complex is formed and stimulates hepcidin production, likely through BMP/SMAD signaling [25]. Specifically, BMP6 is an iron-specific ligand that induces hepcidin production. The disruption of BMP6 activity in mice interrupted this hepcidin production, causing severe iron overload [26]. Just as hepcidin production is increased in times of high iron levels, hepcidin production is suppressed during times of intense iron utilization. Although the mechanisms for this suppression have been incompletely described, it has been observed that erythropoiesis appears to cause the inhibition of hepcidin transcription [27]. This is demonstrated in patients with thalassemia syndromes, who have low hepcidin levels despite the presence of iron overload [28].

Hepcidin regulation by inflammation

Hepcidin production is also modulated by inflammation (Figure 1-5). In mice, inflammatory stimuli including Freund's adjuvant, turpentine, LPS, and heat-killed *Brucella abortus* [29-32] have been shown to increase hepcidin mRNA expression. The primary player in the molecular mechanism of inflammation-induced hepcidin production appears to be the inflammatory cytokine IL-6. A single infusion of IL-6 into healthy human subjects increased hepcidin production and decreased serum iron [30]. This effect has been found to be mediated by the activation of STAT3 (signal transducer and activator of transcription 3). In the presence of inflammation with increased IL-6, STAT 3 undergoes activation and hepcidin promoter binding [33]. There is some indication that other cytokines may also play a role in the activation of hepcidin production. For example, IL-1 has been shown to be stimulate hepcidin production by mouse hepatocytes [34]. However, these findings have not been borne out in human studies [35].

ANEMIA OF INFLAMMATION

Anemia of inflammation (AI), also known as anemia of chronic disease, is a complicating factor of a large spectrum of inflammatory disorders, including chronic renal failure, autoimmune diseases, infections, and certain cancers [36]. While the phenomenon of AI has been observed for many years, it is only recently that the discovery of the peptide hormone hepcidin has helped to elucidate the mechanism of AI development. AI has several typical features: a mild to moderate and normocytic anemia, a shortened erythrocyte lifespan, and depressed erythropoiesis [37]. Perhaps the most characteristic feature of AI is a dysregulation of systemic iron homeostasis manifested by hypoferremia despite intact tissue iron stores [36], resulting in reduced iron availability for erythropoiesis. A common hypothesis for the evolution of this phenotype is that reduced iron availability during times of infection would effectively starve iron-dependent microbes.

The mechanisms for the development of AI are still being elucidated, but there are multiple known contributing factors. The increased production of cytokines, particularly IL-6, in times of inflammation increases hepcidin production, which leads to impaired iron release into the circulation [38]. Reduced iron availability leads to the substitution of zinc for iron during times of iron restriction, and this can be measured by zinc protoporphyrin (ZPP) [39]. In multiple mouse models of inflammation, we have found consistently elevated levels of ZPP in the blood. Other cytokines exert a negative effect on the proliferation of erythroid progenitor cells. Notably, one study found that IFN-γ production stimulates monocytic differentiation at the expense of erythroid differentiation [40]. Evolutionarily, an increase in macrophage numbers would be helpful in eliminating infection. The shortened erythrocyte lifespan that is observed in inflammation could have multiple etiologies. Fibrin deposition on vascular endothelium can lead

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to damaged erythrocyte membranes, and/or macrophage activation by cytokines can cause increased phagocytosis of erythrocytes [40]. Finally, AI is also characterized by reduced erythropoiesis that can occur in the context of either decreased erythropoietin levels and/or impaired responsiveness to adequate levels of circulating erythropoietin [13].

Traditionally, the preferred target in treating AI is the underlying disease process itself. Obviously, this is not always achievable, and alternative therapies include red blood cell transfusion, erythropoiesis-stimulating agents, and iron supplementation. These therapies have been used with varying levels of effectiveness and safety.

Anemia of Aging

Anemia is a common phenomenon in aging humans, affecting about 10% of Americans aged 65 years or older [41]. Anemia of aging (AA) is associated with significant health risks, including impaired mobility [42], cognitive decline [43], increased frequency of hospitalizations [44], and increased mortality [45].

The etiology is multifactorial, and may include nutritional deficiencies, bleeding, and/or chronic inflammation related to chronic kidney disease, malignancy, or infection. However, about a third of AA remains unexplained. One of the proposed mechanisms of unexplained AA relates to IL-6 and hepcidin increase. A well-known phenomenon of normal aging is increased levels of circulating IL-6, which could be secondary to T cell dysfunction [46]. Such an increase would chronically stimulate hepcidin production, leading to a phenotype consistent with AI. Alternatively, IL-6-independent increases in hepcidin can occur secondary to stimulation by other cytokines, or by impaired excretion of the peptide hormone by the kidneys [47]. Further

research is necessary to elucidate the roles of IL-6 and hepcidin in the development of unexplained AI.

Anemia of Cancer

Anemia is exceedingly common in cancer patients, occurring in >30% of cases [48]. Clinical cancer research is a field in which quality of life (QOL) parameters are often considered just as important as mortality statistics, and multiple studies indeed show that even a mild anemia of cancer (AC) is associated with worse QOL parameters [49]. Alternatively, reversal of this anemia has been associated with improved QOL regardless of partial or complete response to chemotherapy [50]. In addition to affecting QOL parameters, AC has been shown to be an independent negative prognostic factor in mortality. One study that evaluated the effect of anemia on mortality in various malignancies found an estimated overall relative risk increase of 65% [51].

The etiology of AC is multifactorial and includes inflammation, nutritional deficiencies, cytotoxic therapies, and bleeding. Because of the chronicity of disease and the associated inflammatory states, AC has been looked at as a variant of AI. Indeed, IL-6 levels have been associated with severity of disease in patients with melanoma [52], non-small cell lung cancer [53], and ovarian cancer [54]. Other cancers, including Hodgkin's disease [55] and multiple myeloma [56], have been shown to have increased hepcidin production with a resulting phenotype that resembles AI. Further studies are necessary to investigate the roles of inflammation and hepcidin in AC.

Anemia of Critical Illness

Another subset of AI is anemia of critical illness (aka ICU anemia), which occurs in the context of overwhelming acute or subacute inflammation. ICU anemia occurs most commonly secondary to sepsis or overwhelming infection. Another common cause is an exacerbation of a chronic autoimmune disorder. The incidence of anemia in intensive care unit (ICU) patients is staggeringly high at >60% [57], and one study found that the average hemoglobin of their septic ICU patients at the time of admission was 10.4 g/dL (normal ~15g/dL) [58]. Similarly to the above anemia syndromes, ICU anemia has been associated with increased morbidity and mortality. One study found that lower hemoglobin was associated with higher Sequential Organ Failure Assessment (SOFA) scores, longer stay, and increased mortality [58].

ICU anemia manifests similarly to AI, with normocytic anemia, shortened erythrocyte lifespan, impaired erythropoiesis, and intact iron stores as evidenced by elevated ferritin levels. As ICU anemia can be considered to be an accelerated form of AI, there is some interest in investigating the role of hepcidin in the mechanism of ICU anemia. One study of 150 trauma patients admitted to the ICU found extremely high levels of urinary hepcidin that correlated with the severity of the injury [59].

Despite its prevalence and significance, AC and ICU anemia are likely undertreated because of the paucity of safe and effective therapies. Red blood cell transfusions carry the risks of immunosuppressive effects, transmission of infections, and transfusion reactions [60], and has been shown to be an independent risk factor for increased mortality in ICU patients [61]. Erythropoiesis-stimulating agents (ESAs) has been associated with higher mortality rates in patients with AC [62], and with deep venous thromboses in ICU patients with negligible decrease in transfusion requirements [63].

SUMMARY

This dissertation provides detailed characterizations of different mouse models in order to characterize the inflammatory anemias that occur secondary to aging, cancer, and sepsis. We examine the roles of inflammation and hepcidin in these models of AI, and lay the groundwork for future research in potential AI therapeutics.

Chapter 2 is a published article that explores the use of a mouse model of aging in order to investigate the mechanism of AA development. Wild-type, IL-6 knockout, and hepcidin knockout mice were aged for up to 24 months, and their inflammatory profiles and hematologic parameters were followed. While all three mice strains developed impaired erythropoiesis, the IL-6 KO and hepcidin KO mice were partially protected. This mouse model demonstrated that hepcidin and IL-6 contribute to the development of AA.

Chapter 3 is a manuscript in preparation, and describes four different mouse models of anemia of cancer. Rapidly-growing and slow-growing cell lines of four different cancer types were injected into wild-type mice, and their hematologic, iron, and inflammatory parameters were analyzed. Three of the four mouse models developed a classic AI phenotype, while the fourth model developed a frank iron-deficiency anemia. Repeating two of the models in hepcidin KO mice illustrated no significant contribution of hepcidin to the development of AC in our models.

Chapter 4 is a submitted manuscript that provides a detailed characterization of a mouse model of severe AI using an injection of *Brucella abortus*. We were able to describe this model's many classic features of AI, including iron-restricted erythropoiesis, increased tissue iron stores, erythropoietic suppression, and shortened RBC lifespan. Repeating the experiment in hepcidin KO mice showed a reversal in iron restriction and a partial improvement of anemia.
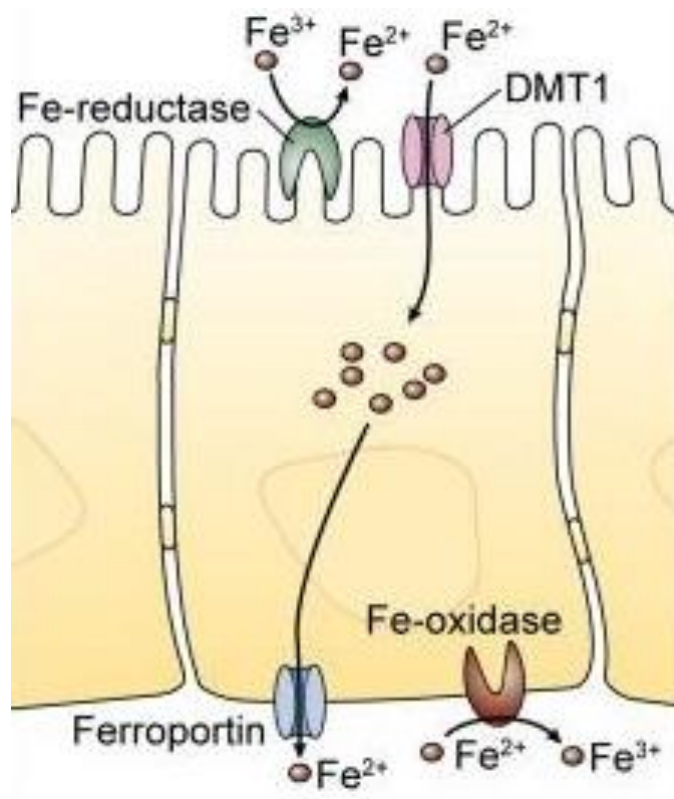
Figure 1-1 (adapted from Andrews 2008) [64]. Iron absorption in the enterocyte.
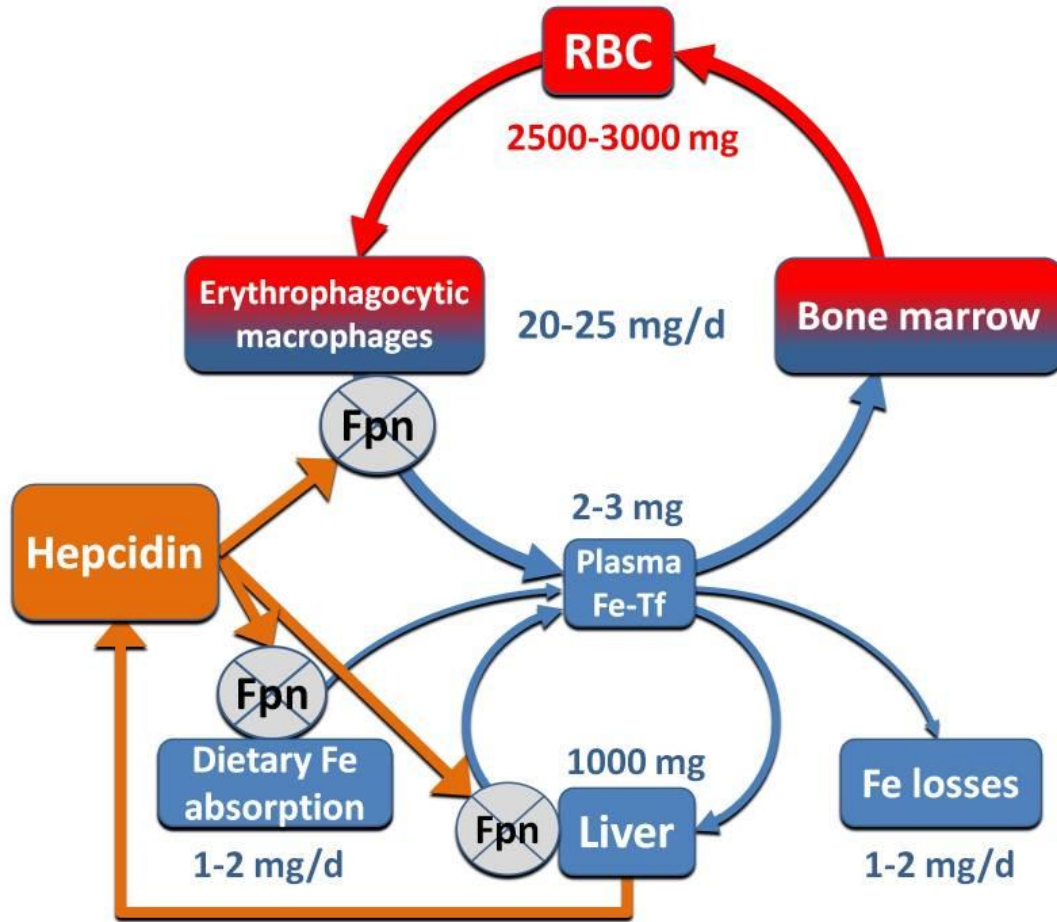
Figure 1-2 [65]. The hepcidin-ferroportin axis regulates systemic iron flow.
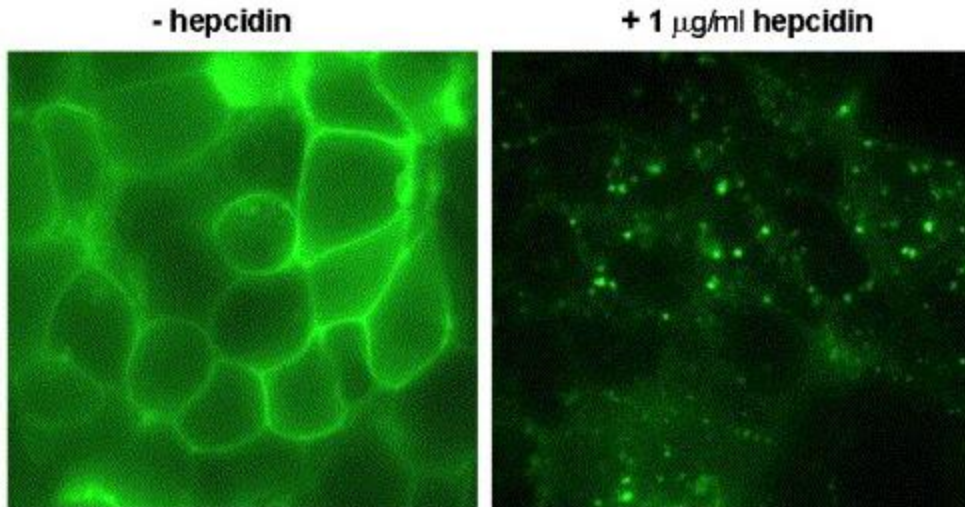
Figure 1-3 [1]. Hepcidin induces internalization and degradation of GFP-tagged ferroportin on HEK293 cells.
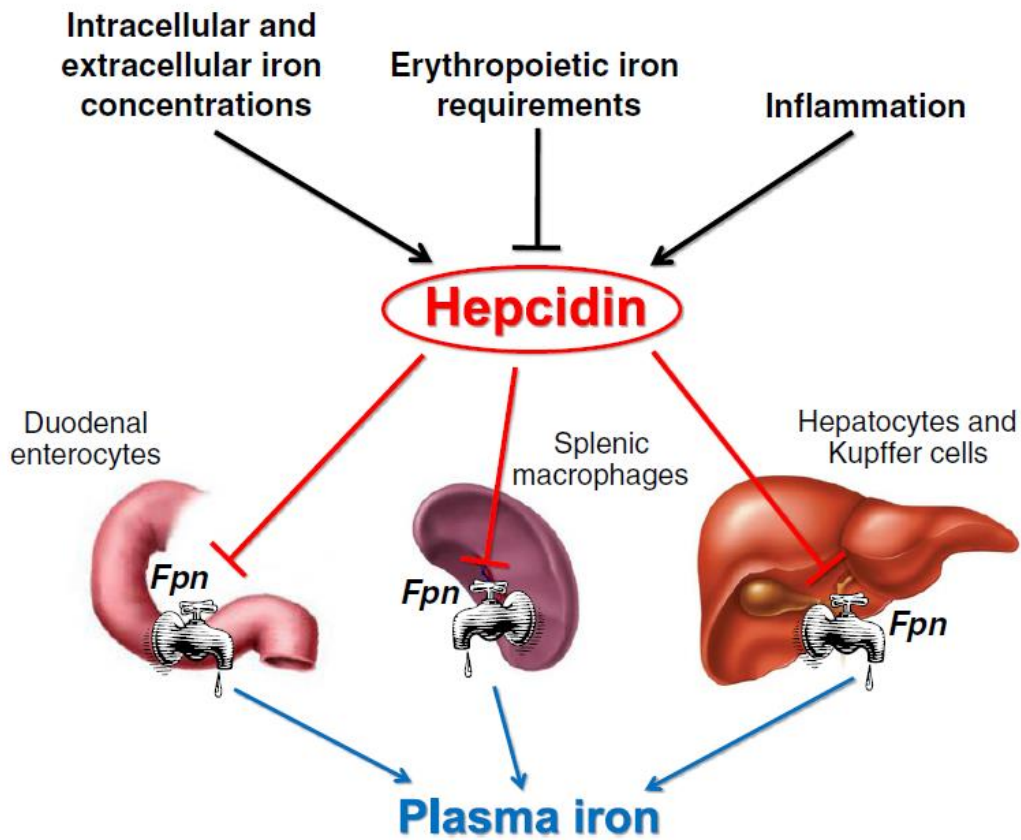
Figure 1-4 [66]. Hepcidin expression is regulated by iron, erythropoiesis, and inflammation. In turn, hepcidin maintains iron homeostasis via its effects on ferroportin activity on enterocytes, macrophages, and hepatocytes.
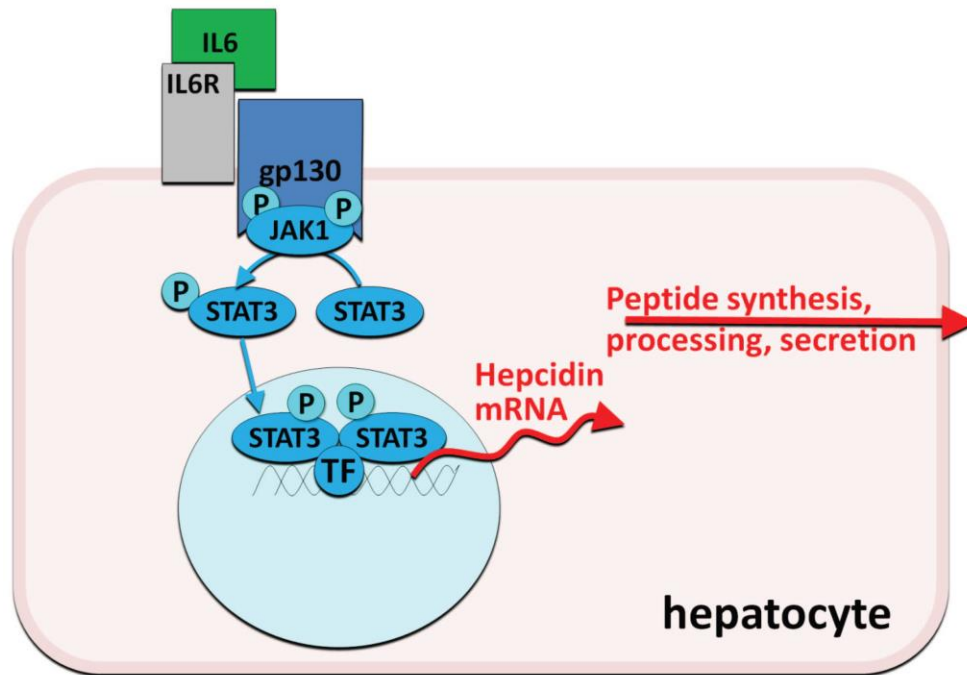
Figure 1-5 [14]. During times of inflammation, hepcidin expression is transcriptionally upregulated via IL-6 activity and the JAK-STAT pathway.

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Chapter 2

INVESTIGATION OF THE ROLE OF INTERLEUKIN-6 AND HEPCIDIN ANTIMICROBIAL PEPTIDE IN THE DEVELOPMENT OF ANEMIA WITH AGE

Investigation of the role of interleukin-6 and hepcidin antimicrobial peptide in the development of anemia with age

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ABSTRACT

Anemia is common in older adults and associated with adverse health outcomes in epidemiological studies. A thorough understanding of the complex pathophysiological mechanisms driving anemia in the elderly is lacking; but inflammation, iron restriction, and impaired erythroid maturation are thought to influence the phenotype. We hypothesized that interleukin-6 contributes to this anemia, given its pro-inflammatory activities, its ability to induce hepcidin antimicrobial peptide, and its negative impact on several tissues in older adults. We tested this hypothesis by comparing changes in indices of inflammation, iron metabolism and erythropoiesis in aged C57BL/6 mice to aged mice with targeted deletions of interleukin-6 or hepcidin antimicrobial peptide. Circulating neutrophil and monocyte numbers and inflammatory cytokines increased with age. Decline in hemoglobin concentration and red blood cell number indicated that C57BL/6, interleukin-6 knockout mice, and hepcidin antimicrobial peptide knockout mice all demonstrated impaired erythropoiesis by 24 months. However, the interleukin-6 knock out genotype and the hepcidin antimicrobial peptide knock out genotype resulted in improved erythropoiesis in aged mice. Increased erythropoietic activity in the spleen suggested that the erythroid compartment was stressed in aged C57BL/6 mice compared to aged interleukin-6 knockout mice. Our data suggest C57BL/6 mice are an appropriate mammalian model for the study of anemia with age. Furthermore, although interleukin-6 and hepcidin antimicrobial peptide are not required, they can participate in the development of anemia in aging mice, and could be targeted, pre-clinically, with existing interventions to determine the feasibility of such agents for the treatment of anemia in older adults.

Introduction

Anemia in older adults affects approximately 10% of community-dwelling adults over 65 years of age and 20% of those over 85 years of age in developed countries.[1-5] Ten to 32%[1,6-8] of anemia cases in adults over 65 years of age are attributable to inflammation. Other major causes include iron deficiency (12-25%[1,6-8] and due to gastrointestinal blood loss in many cases) or hematologic malignancy (6-20%),[6-8] but 26-44% remain unexplained using current clinical diagnostic criteria.[1,6-9] Low hemoglobin has been associated with an increased risk of hospitalization and death,[2,4,10,11] decreased skeletal muscle strength,[3] decreased mobility,[12] and cognitive decline.[13,14] Apart from the effects on the physical well-being of older adults, anemia poses an economic burden to the healthcare system, as it is associated with significant increases in the cost of treating other conditions in the elderly.[15,16]

Cytokine dysregulation has been observed in healthy older adults.[17,18] Interleukin-6 (IL-6) is an extensively studied inflammatory cytokine in aging research.[19] It is a mediator of the acute phase response, participating in the recruitment of neutrophils to sites of injury.[20] IL-6 also participates in the switch from acute to chronic inflammation by binding to its soluble receptor and activating the trans-signaling pathway.[21] Circulating levels of IL-6 become elevated in normal aging.[22,23] Serum IL-6 levels negatively correlate with hemoglobin concentration in chronic disease states,[24,25] but this relationship also exists in frail older adults without overt inflammatory disease.[26] One possible link between IL-6 and erythropoiesis is stimulation of the iron regulatory hormone, hepcidin antimicrobial peptide (Hepc) by IL-6.[27,28] Hepc is hypothesized to be a key driver of the anemia in inflammatory states because it sequesters iron,[29,30] and because it is elevated in the serum of patients with inflammatory conditions and anemia.[31] Hepc may also be elevated independent of IL-6 with age, if it is not effectively cleared by the kidneys,[31] if it is induced by signals from the transforming growth factor β family,[32] or if it is induced by other inflammatory cytokines

such as IL-1α or β.[33] Thus, IL-6 and Hepc may have independent effects on the development of anemia with age.

The molecular pathogenesis of anemia in older adults is poorly understood and optimal clinical interventions have not been adequately defined. Mouse models may be appropriate tools to investigate the pathophysiology of anemia with age and to test possible interventions in a pre-clinical setting, but the pathogenesis of anemia in aged mice has not been adequately investigated. To analyze the molecular and physiological mechanisms that may contribute to anemia in aged mice, we compared indices of inflammation, erythropoiesis, and iron metabolism in 2-year old C57BL/6 wild-type (WT) mice to 2-month old WT mice. To analyze whether IL-6 participates in the development of anemia of aging, we aged for 24 months mice with targeted deletion of IL-6 (IL-6 KO) along with WT mice and assessed the same features of inflammation, erythropoiesis and iron metabolism. To define whether Hepc contributes to impaired erythropoiesis with age, we followed erythrocyte indices in mice with targeted deletion of Hepc (Hepc KO) and WT mice longitudinally from approximately 12 to 24 months. These standard genetic approaches allowed us to assess the requirement of IL-6 or Hepc for features of impaired erythropoiesis in old age. Our results suggest that the anemia which develops in aged C57BL/6 mice is multifactorial. IL-6 and Hepc act as significant and clinically tractable modifiers of the anemia associated with aging in mice, although neither is required for impaired erythropoiesis with age.

Methods

Animal care

The Johns Hopkins University (JHU) Animal Care and Use Committee or the University of California at Los Angeles (UCLA) Animal Research Committee approved all mouse procedures which conformed to applicable laws and guidelines. Retired female breeder (RB) C57BL/6 (WT) or B6.129S2-Il6tm1Kopf/J,[34] (stock 2650, IL-6 KO) were purchased from the Jackson Laboratory at 6-9 months of age and aged to 24 months in the JHU facility (*Online Supplementary Table S1*). Virgin female (VF) WT or IL-6 KO mice (Table 2) were born in the JHU colony to breeders purchased from the Jackson Laboratory, then aged to 24 months. Mice with targeted deletion of the Hepcidin 1 gene[35] were backcrossed ten generations onto the C57BL/6 background (Hepc KO) and aged in the UCLA colony along with their WT controls (Table 3).

Hematologic and iron parameters

Complete blood count was determined using the Hemavet 950FS instrument (Drew Scientific, Waterbury, CT, USA) on whole blood samples collected from the facial vein or retro orbital sinus. Non-heme tissue iron was analyzed using bathophenanthroline, a colorimetric reagent, as described.[36] Serum iron was determined using the Ferrozine-based Iron/TIBC Reagent Set (Pointe Scientific Inc, Canton, MI, USA). The manufacturer's protocol was amended to use 1mL of iron buffer reagent and 50 μL of serum. Serum creatinine was measured by the VetAce® (Alfa Wasserman Diagnostic Technologies LLC, West Caldwell, NJ, USA).

Enzyme-linked immunosorbent assays

Frozen serum samples were thawed once, then analyzed for interferon gamma (IFNγ), IL-1β, IL-10, IL-12p70, IL-6, ker-

atinocyte-derived cytokine (KC), and tumor necrosis factor alpha (TNFα), by the Clinical Research Unit of the Johns Hopkins Institute for Clinical and Translational Research using multiplex analysis (Meso Scale Discovery, Gaithersburg, MD, USA) as described.[37] Erythropoietin was measured by single-plex ELISA (R&D Systems, Minneapolis, MN, USA) as described.[37]

Quantitative real-time PCR

Total liver RNA was analyzed for expression of hepcidin regulatory pathway genes: Hepc; *transmembrane protease, serine 6* (*Tmprss6*); *bone morphogenetic protein 6* (*Bmp6*); and *Inhibitor of DNA binding 1* (*Id1*). Gene expression was normalized to three distinct housekeeping genes: *phosphoglycerate kinase 1* (*Pgk1*); *glyceraldehyde-3 phosphate dehydrogenase* (*Gapdh*); *beta actin* (*Actb*)].

Flow cytometry

Erythroid maturation was determined in bone marrow and spleen as described.[37] We divided Ter119 positive erythroid progenitors into developmental stages I through V[38] using CD44 and forward scatter. Flow Jo software (Tree Star, Ashland,OR, USA) was used to determine frequencies of erythroid progenitors in bone marrow and spleen as well as to determine the median fluorescence intensity of CD71 in individual progenitor populations.

Statistical analyses

Statistical analyses were performed to study differences between 2-month and 24-month, WT or IL-6 KO, mice using Student's t-test with unequal variances. Genotype by age interactions were assessed using 2-way factorial ANOVA. Paired t-test was used to assess the longitudinal changes in hematologic parameters of Hepc KO *vs.* WT mice. For cytokine analyses, the data were log transformed to approximate normality before analysis. Statistical significance was determined as $P<0.05$.

Further information concerning methods is provided in the *Online Supplementary Appendix*.

Results

Aged C57BL/6 mice develop anemia and features of inflammation

To determine whether wild type C57BL/6 (WT) mice developed features of anemia with age, we compared erythrocyte parameters of WT female mice at two months of age to those of WT female retired breeder (RB) mice obtained from the Jackson Laboratory at 6-9 months of age and then aged to 24 months in the JHU facility. Here, we define 'anemia' as a statistically significant lower hemoglobin concentration than the young control group of the same genotype housed in the same facility. Hemoglobin concentration was significantly lower in 24-month old RB mice than in 2-month old mice. Erythrocyte number was significantly lower in 24-month old RB WT mice compared to 2-month old WT mice (Table 1).

Beyond changes in erythrocyte parameters, numbers of neutrophils, monocytes and platelets were significantly increased in aged WT mice (Table 1). Furthermore, the inflammatory cytokines IL-6 and interferon gamma (IFNγ) were significantly increased in aged WT mice (Table 4). We did not detect significant increases with age in any of the other cytokines tested, i.e. IL-1β, IL-10, IL-12p70, keratinocyte-derived cytokine (KC), and tumor necrosis factor alpha (TNFα). Increases in these parameters are consistent with an inflammatory phenotype in the aged WT mice. However, the mice were specified pathogen-free

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Table 1. Cross-sectional analysis of hematologic parameters for aged retired breeder female mice (JHU facility).

Parameter[δ]	2m WT N=54	9-13m WT N=20	24m WT N=16	2m IL-6 KO N=9	9-13m IL-6 KO N=30	24m IL-6 KO N=16	Genotype x Age P-value[Δ]
Neutrophils (K/µL)	0.44±0.44	2.11±0.94	1.13±0.70*	0.44±0.21	1.78±0.90	1.09±0.72^	0.86
Monocytes (K/µL)	0.17±0.14	0.46±0.21	0.30±0.13*	0.08±0.04[†]	0.36±0.12	0.32±0.36^	0.25
Lymphocytes (K/µL)	3.19±1.93	6.41±2.56	1.74±0.95*	2.19±0.87[†]	5.54±1.54	2.45±1.16	0.04
Erythrocytes (M/µL)	9.56±0.45	9.21±0.96	7.83±1.45*	9.59±0.24	9.64±1.09	8.84±0.43^	0.01
Hemoglobin (g/dL)	13.7±0.8	13.8±1.1	10.8±1.8*	13.9±0.51	14.1±1.5	12.0±0.9^	0.04
MCV (fL)	45.6±1.3	44.2±1.8	43.9±4.6	46.7±0.9[†]	44.2±2.6	44.2±4.1^	0.53
MCH (pg)	14.4±0.6	15.1±0.9	13.9±1.4	14.4±0.3	14.7±0.8	13.6±0.6^	0.26
RDW (%)	17.3±0.6	19.3±2.9	18.4±1.4*	16.7±0.5[†]	17.7±1.5	17.2±0.6^	0.18
Platelets (K/µL)	753±90	1150±322	1025±270*	743±47	1086±304	938±178^	0.31

[δ]±Standard Deviation; P≤0.05, 2-sample t-test with unequal variance: *2m WT vs. 24m WT; [†]2m WT vs. 2m IL-6 KO; ^2m IL-6 KO vs. 24m IL-6 KO; [Δ]2-way factorial ANOVA.

Table 2. Cross-sectional analysis of hematologic parameters for aged virgin female mice (JHU facility).

Parameter[δ]	2m WT N=54	9-13m WT N=34	24m WT N=25	2m IL-6 KO N=9	9-13m IL-6 KO N=15	24m IL-6 KO N=8	Genotype x Age P-value[Δ]
Neutrophils (K/µL)	0.44±0.44	1.48±0.49	1.19±0.88*	0.44±0.21	1.19±0.48	0.90±0.42^	0.35
Monocytes (K/µL)	0.17±0.14	0.40±0.16	0.38±0.35*	0.08±0.04[†]	0.43±0.21	0.39±0.21^	0.37
Lymphocytes (K/µL)	3.19±1.93	5.53±1.77	2.23±1.36*	2.19±0.87[†]	5.38±2.09	3.70±2.69	0.01
Erythrocytes (M/µL)	9.56±0.45	9.99±0.46	8.68±0.99*	9.59±0.24	9.87±0.44	9.08±0.66	0.29
Hemoglobin (g/dL)	13.7±0.8	14.3±0.8	12.0±1.2*	13.9±0.51	14.4±0.44	12.4±0.7^	0.61
MCV (fL)	45.6±1.3	44.9±2.2	43.5±4.0*	46.7±0.9[†]	44.9±1.5	41.4±1.9^	0.01
MCH (pg)	14.4±0.6	14.3±0.7	13.9±0.9*	14.4±0.3	14.6±0.5	13.7±0.7^	0.41
RDW (%)	17.3±0.6	18.6±0.6	18.0±1.2*	16.7±0.5[†]	18.1±0.9	17.8±0.4^	0.31
Platelets (K/µL)	753±90	904±197	1100±203*	743±47	843±201	889±151^	0.01

[δ]± Standard Deviation; P≤0.05, 2-sample t-test with unequal variance, *2m WT vs. 24m WT; [†]2m WT vs. 2m IL-6 KO; ^2m IL-6 KO vs. 24m IL-6 KO; [Δ]2-way factorial ANOVA.

and had no obvious tumors or lesions that would indicate overt inflammatory disease. In contrast to increased neutrophils and monocytes, lymphocytes were significantly decreased in 24-month RB WT mice (Table 1). This skewing of immune cells, toward the myeloid lineage and away from the lymphoid lineage, is consistent with an aging hematopoietic compartment[39,40] and the pro-inflammatory state of aging that has been described for older adults.[41,42]

We were concerned that the RB females may experience anemia or inflammation due to repeated physical stress, tissue injury, remodeling and repair associated with birthing pups. To determine whether virgin female (VF) mice would develop anemia with age like the RB females, we compared erythrocyte parameters of female WT mice at two months of age to those of VF WT mice born and aged to 24 months in the JHU facility (Table 2 and *Online Supplementary Table S1*). Hemoglobin concentration was significantly lower in 24-month old VF mice compared to 2-month old mice (Table 2). Since VF WT mice that had never given birth to pups also developed anemia with age, we concluded that anemia in aged female C57BL/6 mice is a general finding that is not restricted to the RB WT mice. Like RB WT, 24-month old VF WT mice also developed a phenotype of myeloid skewing. Neutrophils, monocytes, and platelets were all elevated while lymphocytes were significantly decreased in 24-month old VF WT mice (Table 2).
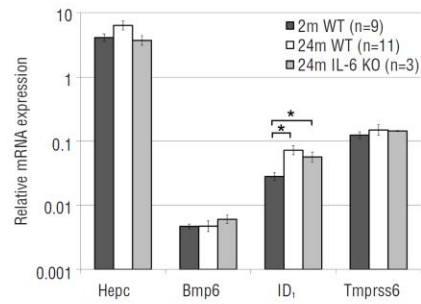
Figure 1. Relative mRNA expression of members of the Hepc regulatory pathway in the liver of young and aged WT and aged IL-6 KO mice. We assessed the fold change relative to internal housekeeping genes for each target gene and each group of mice. This relative mRNA expression of 2 month (m) WT (dark gray bars), 24m WT (white bars), and 24m IL-6 KO (light gray bars) mice is plotted in log scale. We found a non-significant trend toward increased (54%) Hepc mRNA levels in 24m WT mice over 2m WT mice (P=0.060). We observed a significant increase in Id1 mRNA levels in 24m WT (2.57 fold; P<0.001) and 24m IL-6 KO (2.0 fold; P=0.023) mice compared to 2m WT mice. We found no significant change in expression of Bmp6 despite a significant increase in non-heme liver iron concentration with age. Similarly, we found no significant change in Tmprss6 expression. The error bars were determined by calculating the standard error of the mean (SEM) for ΔCt for each gene and group and then converting to relative mRNA expression (2-average ΔCt ± SEM). Asterisk indicates P<0.05.

Targeted deletion of IL-6 modifies anemia in aged retired breeder female mice

Since aged WT mice developed anemia and features of inflammation, we tested the hypothesis that IL-6, a central mediator of chronic inflammation, is required for the development of anemia in aged mice. We compared erythrocyte parameters of female IL-6 KO mice at 2 months of age to 24 month old female RB IL-6 KO mice obtained from the Jackson Laboratory at 6-9 months of age and then aged to 24 months in the JHU facility (Table 1). Hemoglobin was significantly lower in the aged RB IL-6 KO *versus* young IL-6 KO mice, but this change was significantly smaller in magnitude than the decline of WT RB mice with age (Table 1, *P*=0.04 for genotype by age interaction). Additionally, the variability in hemoglobin concentration was smaller in the aged RB IL-6 KO mice than the aged RB WT mice (Table 1). Though erythrocyte number was lower in aged RB IL-6 KO mice than young, the decline in red blood cell number for aged IL-6 KO RB mice was not as severe as the decline for aged WT RB mice (Table 1; *P*=0.01 for genotype by age interaction). A longitudinal study of IL-6 KO mice in the UCLA facility also demonstrated that aged IL-6 KO mice develop less severe anemia than their WT counterparts (*P*=0.016; *Online Supplementary Table S2*). Aged IL-6 KO RB mice had features of inflammation similar to those of aged RB WT mice, including increased neutrophils, monocytes and platelets compared to their 2-month old counterparts. IL-6 KO mice have a known lymphopoietic defect,[34] but IL-6 KO mice were protected from further reduction in circulating lymphocyte numbers with age (Table 1; *P*=0.04 for genotype by age interaction).

We performed similar analyses for VF IL-6 KO mice born and aged to 24 months in our JHU facility (Table 2). Like their RB IL-6 KO counterparts, aged VF IL-6 KO mice also had increased neutrophils, monocytes and platelets compared to 2-month controls. Like their RB IL-6 KO counterparts, aged VF IL-6 KO mice were protected from further decline in lymphocyte production (Table 2; *P*=0.01 for genotype by age interaction). We found hemoglobin was significantly lower in the aged VF IL-6 KO mice than 2-month IL-6 KO mice (Table 2). However, there was no difference in the magnitude of the reduction in hemoglobin with age between VF WT mice and VF IL-6 KO mice (Table 2; *P*=0.61 for genotype by age interaction). This suggested to us that the protective effect of the IL-6 KO genotype on the development of anemia with age in the JHU facility was limited to RB mice.

Targeted deletion of hepcidin modifies anemia in aged mice

Hepc is a central regulator of iron homeostasis and a critical mediator of anemia in some inflammatory states.[43] We tested the hypothesis that Hepc is required for the development of impaired erythropoiesis in aged mice. We followed erythrocyte parameters in peripheral blood of Hepc KO mice longitudinally from 12-13 months to 17-23 months of age in our UCLA facility (Table 3). Hepc KO mice are severely iron loaded.[35] This excess iron may contribute to the significant increase in erythrocytes and hemoglobin in Hepc KO mice at the 12-13 month baseline when compared to WT mice in the same facility. However, increased erythrocytes and hemoglobin were not observed in 2-month Hepc KO mice in a previous study.[44] Hemoglobin and erythrocyte number declined significantly in mice of both genotypes with age, but the magnitude of the reduction in hemoglobin was significantly less for aging Hepc KO mice than for WT mice (Table 3; *P*=0.002 for genotype by age interaction). The effect of the Hepc KO genotype on hemoglobin decline with age was only statistically significant in the male mice (*Online Supplementary Table S3*), but the female mice demonstrated the same trend (*Online Supplementary Table S4*).
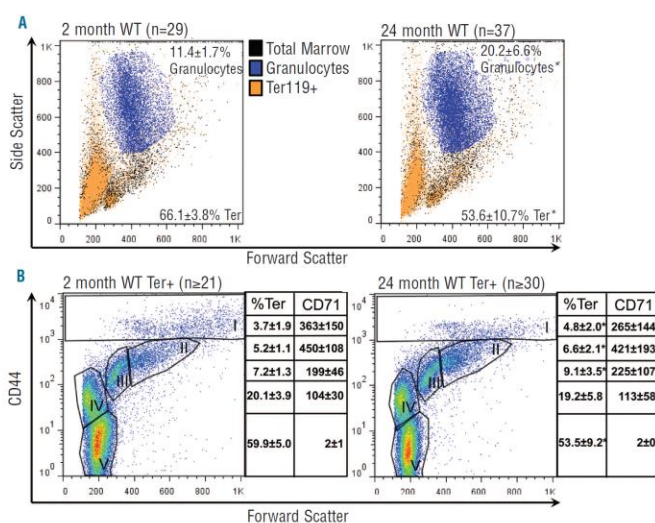
Figure 2. Erythroid maturation is impaired in aged WT mice. (A) We assessed the percentage of erythroid progenitors [Ter119 positive (Ter), back gate appears in orange] in the bone marrow of 2m (left panel) or 24m (right panel) WT mice. We found granulocyte progenitors (events with high forward scatter and high side scatter appear in blue) were significantly increased in aged mice, while Ter+ progenitors were significantly decreased. Values are expressed as mean ± standard deviation. Asterisk indicates *P*<0.001. (B) Total bone marrow cells were selected for Ter119 to define committed erythroid progenitors. We found a statistically significant increase in the percentage of early stage (CD44high: I, II, and III) progenitors in the aged mice (right panel) and a significant decrease in the percentage of latest stage erythrocytes (CD44low). We observed no difference in median CD71/TfR mean fluorescence intensity among young and aged mice in the various stages of development. Values are expressed as mean ± standard deviation. Significance *P*≤0.05 is indicated with an asterisk and was determined by Student's t-test with unequal variance between groups.

Anemia in aged mice is not iron-restricted

Next, we assessed biochemical features that might account for the anemia in aged WT and IL-6 KO mice (Table 4). We could not detect statistically significant differences in these parameters between aged VF and aged RB mice (*Online Supplementary Table S5*). The differences in parameters in Table 4 between young mice and aged VF mice followed the same trend as the differences between young mice and aged RB. In the light of these observations, we combined the data for aged RB and aged VF. We found no evidence of frank iron deficiency in aged mice. In fact, liver and spleen non-heme iron stores were significantly increased in aged WT mice and aged IL-6 KO mice as compared to their young genotype controls (Table 4).

To assess whether iron loading and inflammation affected genes in the Hepc regulatory pathway in aged mice, we investigated the expression of select hepatic genes. We found a non-significant trend toward increased expression of Hepc in the livers of aged WT mice *versus* young WT mice (Figure 1 and *Online Supplementary Table S6*). Hepc expression was not low, as would be expected in the context of anemia.[31,45] We hypothesized Hepc expression in aged, anemic WT mice may be maintained in the same range as young WT mice either in response to inflammatory signals or in response to iron loading. Liver iron con-

centration is increased 2-fold in aged mice, but we did not observe an increase in Bmp6 expression with age, though it has been demonstrated to respond to iron load. Kautz and colleagues[46] demonstrated less than a 2-fold change in Bmp6 in Hepc KO mice which were shown to have a 5-fold increase in liver iron stores, and mainly in hepatocytes.[35] We speculate that the magnitude and pattern of iron loading in aged mice may not be sufficient to significantly induce Bmp6. We further speculate that other pathways, active in aged mice, may interfere with the Bmp6 response to iron loading. We did observe an increase in Id1 expression, which is sensitive to increased iron stores[46] and IL-6.[47] IL-6 is the primary mediator of increased Hepc expression in response to inflammation,[27] but we were not able to detect a significant decrease in Hepc in our aged IL-6 KO mice (Figure 1 and *Online Supplementary Table S6*).

To test whether aged mice experienced iron-restricted erythropoiesis, we assessed iron availability to the erythron and erythroid iron utilization. Serum iron concentrations were mildly decreased in aged *versus* young WT mice (Table 4). Despite this observation, serum iron was not low enough to induce substantial iron-restricted erythropoiesis. MCV and MCH remained close to the normal range in aged WT mice. To determine whether developing erythroblasts were iron-restricted, we assessed median

Table 3. Longitudinal analysis of hematologic parameters of Hepc KO mice with age (UCLA facility).

Parameter[$]	13-14 m WT n=6F + 4M	21m WT n=6F + 4M	12-13m Hepc KO n=11F + 10M	17-23m Hepc KO n=11F + 10M	Genotype x Age P-value[&]
Erythrocytes (M/L)	9.01±0.48	7.65±0.91*	10.07±0.53[¶]	9.18±1.71[^]	0.375
Hemoglobin (g/dL)	12.1±0.4	8.0±1.3*	14.9±0.8[¶]	12.8±1.7[^]	0.002
MCV (fL)	44.7±1.8	43.5±3.9	48.2±2.4[¶]	47.3±3.8	0.792
Platelets (K/L)	1013±243	1251±494	1144±261[¶]	1005±280	0.041

$± Standard Deviation; *P≤0.05, paired T-test of difference by age (14 m vs. 21m) within WT genotype group; ¶P≤0.05, two-sample T-test of difference between genotypes within age group (14m WT vs. 13m Hepc KO); ^P≤0.05, paired T-test of difference by age (13 m vs. 23m) within Hepc KO genotype group; &two-sample T-test of the difference score between young and old by genotype.

Table 4. Cross-sectional analysis of biochemical features of young and aged (virgin female and retired breeder) WT and IL-6 KO mice (JHU facility).

Parameter	2m WT n≥ 13	24m WT n≥21	2m IL6 KO n≥ 9	24m IL-6 KO n≥ 7	Genotype x Age P-value[&]
IL6 (pg/mL)[@]	7.5±5.4	41.6±2.6*	NA	NA	NA
IFNγ (pg/mL)[@]	0.41±2.25	1.05±2.53*	0.21±3.79	1.75±3.21[^]	0.04
Liver iron (µg/g)[$]	113±25	239±90*	128±24	318±94[^]	0.06
Spleen iron (µg/g)[$]	599±364	1827±977*	788±338	1617±775[^]	0.25
Spleen weight (mg)[$]	55.7±9.1	163.4±73.1*	46.8±9.0[¶]	68.7±18.1[^]	<0.001
Iron per spleen (mg)[$]	33±21	266±146*	33±14	102±48[^]	<0.001
Serum Iron (µg/dL)[$]	168±43	127±39*	140±37	133±34	0.08
Ter+ Marrow (%)[$]	66.1±3.8	53.6±10.7*	67.0±3.9	61.5±7.3[^]	0.03
Ter+ Splenocytes (%)[$]	39.6±4.9	72.8±9.5*	43.7±11.4	53.4±10.6[^]	<0.001
Retics (M/µL)[$]	0.261±0.049	0.368±0.191*	0.238±0.033	0.245±0.034	0.07
Retics (%)[$]	2.72±0.49	5.06±4.45*	2.51±0.39	2.67±0.33	0.08
Epo (pg/mL)[$]	0 (0-146)	0 (0-660)	0 (0-197)	0 (0-131)	NA
Creatinine (mg/dL)[ε]	0.29±0.10	0.29±0.06	ND	0.26±0.09	NA

@Geometric mean ± geometric standard deviation, with corresponding tests performed on the log values of the parameter; $± Standard Deviation; P≤0.05, two sample t-test with unequal variance: *2m WT vs. 24m WT; #2m WT vs. 2m IL-6 KO; ^2m IL-6 KO vs. 24m IL-6 KO; &2-way factorial ANOVA; ε median and (range).

CD71/TfR expression across the stages of erythroblast development (Figure 2). We found no statistically significant increase in median CD71/TfR expression, suggesting iron was not limiting to erythroid precursors.

Impaired erythroid maturation in aged mice

Without strong evidence for iron-restricted erythropoiesis with age, we investigated the development of erythroid progenitor/precursors in aged mice. We could not detect a statistically significant difference in the cellularity of the marrow of aged *versus* young mice. We did observe a significant reduction in the percentage of committed erythroid progenitor/precursors (Ter119 positive) in the marrow of aged mice (Table 4 and Figure 2A). Simultaneously, we found an increase in the percentage of granulocytes (Figure 2A) which is consistent with increased circulating neutrophils and similar to our previous observations in mice with turpentine-induced sterile abscesses.[37]

We observed that aged mice had greater splenic hematopoiesis, possibly to compensate for the limited erythropoiesis in the bone marrow. We found a significant increase in spleen weight in aged WT mice as well as a significant increase in the percentage of splenic erythroid progenitor/precursors (Table 4 and *Online Supplementary Figure S1*). A limitation of these results is that we did not assess markers of erythropoiesis in the spleen using gene expression analyses. Extramedullary hematopoiesis in aged WT mice was not sufficient to normalize hemoglobin concentration.

We further investigated whether the development of erythroid progenitors was impaired (Figure 2). We assessed maturation of erythroid progenitor/precursors in the bone marrow with the use of CD44 (Figure 2B). We found a slight increase in the percentage of early stage (I-III) progenitors and a slight reduction in the percentage of late stage (V) progenitors in aged WT mice (Figure 2B, right panel). These results suggest an inhibition of maturation at early stages of development, or destruction of the latest stages of development that we expect contributes to the reduced erythrocyte numbers in the peripheral blood of aged WT mice (Tables 1 and 2).

IL-6 KO genotype reduces erythropoietic stress

Compared to aged WT mice, aged IL-6 KO mice appeared to have less erythropoietic stress and more intramedullary erythropoiesis. The percentage of erythroid progenitors and precursors in the bone marrow did not decline in aged IL-6 KO mice to the extent of aged WT mice (Table 4; P=0.03 for genotype by age interaction). This increased efficiency of marrow erythropoiesis in IL-6 KO mice may have reduced the need for extramedullary erythropoiesis. Spleen weights increased only 50% in aged IL-6 KO mice, compared to a 3-fold increase in aged WT mice (Table 4; P<0.001 for genotype by age interaction). Additionally, aged WT mice produced greater percentages of reticulocytes and splenic erythroid progenitor/precursors than their IL-6 KO counterparts (Table 4). Aged IL-6 KO mice did not increase splenic iron stores to the same extent as aged WT mice (Table 4; P<0.001, for genotype by age interaction). We hypothesize this difference may be attributed to depletion by more robust aged IL-6 KO erythroid precursors that utilize iron more efficiently than aged WT erythroid precursors. From these data, we conclude that erythropoiesis is less impaired in aged IL-6 KO mice than aged WT mice.

To determine whether the observed differences in erythropoiesis between aged WT and IL-6 KO mice was attributable to erythropoietin (Epo), the primary survival factor for erythroid progenitors, we assessed serum Epo concentrations. Epo was not detectable in the majority of young or aged mice. However, the range of Epo was greatest for aged WT mice (Table 4). Epo is primarily produced by the interstitial cells of the kidney and chronic kidney disease can reduce Epo production significantly. We measured serum creatinine as a surrogate of kidney function, but found no difference in serum creatinine with age (Table 4). Combined, these data suggest greater erythropoiesis in aged IL-6 KO mice is not Epo-dependent.

Discussion

We have demonstrated that C57BL/6 mice are an appropriate mammalian model for the development of anemia with age. We conclude that anemia in aged mice is multifactorial, similar to what has been observed in older adults.[1,6,9] Herein, we investigated features of inflammation, iron restriction, and impaired erythroid development to characterize the anemia of aging. We employed two key mouse models with targeted deletions of IL-6 or Hepc to test whether either molecule was required for impaired erythropoiesis with age.

We demonstrated that both VF and RB WT mice model age-associated inflammation in the absence of an overt inflammatory disease. They have increased circulating neutrophils and monocytes and increased concentrations of IL-6 and IFNγ, compared to young controls. All groups of aged RB and VF mice had significantly lower hemoglobin at 24 months of age than their young, genotype controls. Because IL-6 negatively correlates with hemoglobin in various disease states,[24-26] we tested the hypothesis that IL-6 deficiency could increase hemoglobin at 24 months. Twenty-four month old IL-6 KO RB mice demonstrated anemia, but it was significantly reduced in severity compared to 24-month old WT RB mice. Furthermore, we found aged IL-6 KO mice had less extensive extramedullary erythropoiesis and a greater percentage of committed erythroid progenitors and precursors in the marrow, demonstrating more efficient erythropoiesis.

The pro-anemic effects of the IL-6 pathway were most apparent in RB mice, which experienced greater physiological stress than their VF counterparts due to pregnancy, tissue injury, and repair that occurs with birthing. Increased physiological stress in RB WT mice may contribute to the development of more severe anemia than their VF WT counterparts. In contrast to the effect of the IL-6 KO genotype in RB mice, aged VF IL-6 KO mice did not have significantly increased hemoglobin concentration compared to aged VF WT mice. However, VF mice selected for their features of longevity, resistance to tumors, wounds, and other diseases, and housed in a specified pathogen-free environment are not likely to adequately reflect the physiological stress of the majority of community-dwelling older adults. We conclude that IL-6 reduces the efficiency of erythropoiesis in the bone marrow of aged C57BL/6 mice, resulting in more extensive splenic erythropoiesis that does not fully compensate for the anemia, especially in the context of physiological stress.

Aged WT mice model some features of iron sequestration that may be attributed to inappropriately high *Hepc*

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expression. *Hepc* expression usually declines in the context of anemia[31,45] but was not significantly decreased in anemic aged WT mice. Consistent with increased Hepc activity, serum iron concentrations were lower in aged WT mice than in young WT mice despite higher non-heme splenic iron stores. Importantly, the magnitude of the decline in hemoglobin concentration was reduced in aged Hepc KO mice when compared to that of aged WT mice.

Our analysis of mice with targeted deletions in key genes regulating inflammation and iron homeostasis demonstrates that neither IL-6 nor Hepc is required for the anemia that develops with age. The expansion of granulocytes in the marrow and increased neutrophils and monocytes in the peripheral blood of WT and IL-6 KO mice suggest an overall expansion of myeloid cells, yet erythrocyte numbers decline. Lymphocytes also declined in WT mice. This inflammatory phenotype and impaired erythropoiesis that develops in mice is consistent with the changes in the aging hematopoietic compartment described as "myeloid skewing".[48,49] We also found that IFNγ, which is known to have deleterious effects on hematopoietic stem cells,[50] increased with age.

Though aged mice demonstrated the widest distribution of serum Epo concentrations, Epo was not significantly elevated in the majority of aged mice despite anemia. A similar observation of inappropriately low Epo concentrations has been made for older adults with unexplained anemia.[6,7,9] These observations may implicate a dysregulated hypoxia response with age that further contributes to anemia of aging.

In summary, we have demonstrated anemia in aged C57BL/6 mice is multi-factorial. Though mechanisms independent of Hepc and IL-6 can also impair erythropoiesis with age, future studies should address the use of anemic, aged mice as a pre-clinical model to test the efficacy of interventions targeting Hepc and IL-6 pathways. Additionally, interventions targeting the IFNα pathway or modifiers of erythroid and myeloid progenitor commitment may be appropriate for pre-clinical testing.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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Chapter 3

FOUR MOUSE MODELS ILLUSTRATE DIFFERENT

MECHANISMS OF ANEMIA OF CANCER

Manuscript in preparation:

Four Mouse Models Illustrate Different Mechanisms of Anemia of Cancer

ABSTRACT

The anemia of cancer (AC) is an undertreated complication of cancer that has been shown to adversely affect quality of life. Although there are many purported mechanisms of AC, there are few tools to study its pathogenesis. We have characterized four mouse models to study the development of AC in various cancers of varying chronicities. Our rapidly-growing melanoma model developed a severe anemia by 14 days, with hematologic and inflammatory parameters consistent with anemia of inflammation. While both of our rapidly-growing lung cancer models developed the characteristic findings of AI, they differed greatly in the severity of anemia. Interestingly, elevation in hepcidin mRNA levels of the milder lung cancer model pointed to a possible role of hepcidin in the development of anemia in these mice. Our slow-growing model of ovarian cancer developed a frank iron-deficiency anemia, likely secondary to hemorrhagic ascites and nutritional deficiencies. Ablation of hepcidin in two of the above mouse models resulted in no difference in the severity of anemia. While the elevation of hepcidin mRNA in the lung cancer model had suggested a role of hepcidin in the development of AC, the lack of anemia attenuation with hepcidin ablation pointed to hepcidin-independent factors in anemia development in these mice.

INTRODUCTION

Anemia is a strikingly common complication in cancer patients, occurring in >30% of patients [1]. While anemia of cancer (AC) is often overlooked as a relatively mild consequence of malignancy, studies show a strong positive correlation between AC, and WHO performance status and quality of life [2, 3]. In fact, the effective treatment of AC is an important intervention to decrease the complaint of fatigue in cancer patients [4]. In addition to the effects on quality-of-life parameters, the presence of AC is a known independent negative prognostic factor [5].

The etiology of AC is multifactorial and likely varies with the primary tumor, as well as the chronicity of the disease process. Important contributing factors include iron deficiency secondary to bleeding or nutritional deficiencies [6], hemolysis [7], and impaired erythropoiesis secondary to impaired erythropoietin production or depressed response of the erythroid marrow to erythropoietin [8]. A particularly important contributor to AC is inflammation, as AC shares many of the characteristics of anemia of inflammation (AI). AI is a normocytic, normochromic anemia with a shortened erythrocyte lifespan and depressed erythropoiesis. AI is also characterized by a derangement of systemic iron homeostasis characterized by hypoferremia with intact iron stores [9] and decreased availability of iron for erythrocyte production.

Hepcidin, a 25-amino acid peptide hormone produced primarily by hepatocytes [10], is the primary regulator of iron homeostasis in health and during inflammation [11]. Hepcidin acts by binding ferroportin, the sole known cellular iron exporter, displayed on the surface of macrophages, hepatocytes, and the basolateral membranes of enterocytes. Hepcidin binding to ferroportin leads to ferroportin endocytosis and degradation [12]. During inflammation, hepcidin expression is strongly induced, largely by IL-6 [13] via the JAK-STAT pathway [14-16].

Interestingly, IL-6 has been noted to be increased in multiple tumor types, including lung cancer [17] and ovarian cancer [18]. The extent to which increased hepcidin production contributes to AC has not been directly tested by genetic ablation of hepcidin, partially because of the paucity of mouse models of AC.

Despite the high prevalence of AC with its negative effects on quality of life and survival, few animal models have been developed for its study. We have characterized four mouse models of cancer: two rapidly-growing models of lung cancer, one rapidly-growing model of melanoma, and one slow-growing model of ovarian cancer. After a comprehensive examination of hematologic, iron, and inflammatory parameters of these models, we investigated the extent of hepcidin involvement in the development of AC in these models by comparing C57Bl/6 mice to hepcidin$^{-/-}$ mice on the same strain background.

MATERIALS AND METHODS

Animal models of anemia of cancer

All animal studies were approved by the Animal Research Committee at University of California, Los Angeles (UCLA). 6-week-old male and female C57BL/6J mice were obtained from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratories (Bar Harbor, ME). Wild-type (WT) mice were started on a low-iron diet (20 ppm iron, Harlan Teklad, Indianapolis, IN) for 2 weeks before injection of tumor cells or saline. The same low-iron diet was used through the remainder of the experiment. This dietary conditioning was applied because high iron content of standard chow dramatically upregulates hepcidin expression and renders it unresponsive to inflammatory stimuli [19]. Additionally, dietary iron absorption in mice accounts for as much as 50% of the daily iron fluxes in mice fed standard chow, but only ~5-10% in humans [20]. This increased dietary iron absorption in mice may obscure the contribution of iron recycling by macrophages [21] and leads to progressive iron loading. Thus, the reduction of dietary iron content in mice chow models iron fluxes of human homeostasis.

To induce anemia of melanoma, animals were injected intraperitoneally (IP) with 0.1×10^6-0.3×10^6 B16-F10 cells (ATCC, Manassas, VA). The B16-F10 cells were cultured as adherent cells in Dulbecco's modified Eagle media (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G-streptomycin. On the day of treatment, tumor cells were resuspended in PBS, counted with a hematocytometer, and injected in a volume of 500 μL phosphate buffered saline (PBS). Control mice were injected IP with 500 μL PBS. Mice were euthanized at 14 days, and blood and liver were collected at necropsy.

To induce anemia of metastatic lung cancer, animals were injected IP with 0.1×10^6-0.5×10^6 murine TC-1 or Lewis lung carcinoma (LLC) cells (ATCC, Manassas, VA). The LLC cells were cultured as adherent cells in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS and 1% penicillin G-streptomycin, and the TC-1 cells were cultured as adherent cells in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FBS and 1% penicillin G-streptomycin. On the day of treatment, tumor cells were resuspended in PBS, counted with a hematocytometer, and injected in a volume of 500 µL PBS. Control mice were injected IP with 500 µL PBS. Mice were euthanized at 13-15 days, and blood, liver, and spleen were collected at necropsy.

To induce anemia of ovarian cancer, animals were injected IP with 1×10^6 ID8 cells that were generously provided by Dr. Oliver Dorigo. The metastatic ID8 cell line was derived from spontaneous malignant transformation of C57/Bl6 mouse ovarian surface epithelium cells *in vitro* [22]. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin G-streptomycin. On the day of treatment, tumor cells were resuspended in PBS, counted with a hematocytometer, and injected in a volume of 500 µL PBS. Control mice were injected IP with 500 µL PBS. Mice were euthanized at 18.5 weeks.

To study the role of hepcidin in anemia of cancer, we used male and female hepcidin-1 knockout mice (*Hamp-/-*). *Hamp-/-* mice were originally provided to our laboratory by Dr. Sophie Vaulont [23] and were backcrossed onto the C57Bl/6 background as previously described [24]. For this portion of the study, *Hamp-/-* mice underwent dietary conditioning to prevent the development of iron overload and maintain iron levels comparable to those of WT mice. At weaning, the

Hamp1-/- mice were started on low-iron diets (4 ppm or 20 ppm) for about 2 weeks. From the time of injection, the mice were maintained on a 20 ppm iron diet for the remainder of the study. For the TC-1 model, *Hamp1-/-* mice were injected and euthanized as described above for the WT mice. For the ID8 model, *Hamp1-/-* mice were euthanized at 16.5 weeks.

Measurement of iron parameters and erythropoietin

Serum iron and liver non-heme iron concentrations were measured by a colorimetric assay for iron quantification (Sekisui Diagnostics; Lexington, MA) as previously described [20].

Hematologic studies

Complete blood counts were obtained with a HemaVet blood analyzer (Drew Scientific; Waterbury, CT). To assess iron-restricted erythropoiesis, zinc protoporphyrin (ZPP) was measured using a hematofluorometer (AVIV; Lakewood, NJ). Reticulocytes were counted by flow cytometry. Blood (5 μl) was added to 1 ml of thiazole orange in PBS with 0.1% sodium azide (PBS-azide, BD Bioscience; San Jose, CA) and incubated at room temperature for 1-3 h. As an unstained control, blood was added to PBS-azide without thiazole orange. The percentage of red-fluorescent reticulocytes (Retic %), was measured by flow cytometry at the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards CA-16042 and AI-28697, and by the JCCC, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA. Unstained controls were used to establish a gate to exclude background fluorescence. The results are expressed as the reticulocyte product index: RPI = Retic % x Hgb / baseline Hgb.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from liver and analyzed by real-time RT-PCR as described previously [25].

Primers are listed in Supplemental Table 3-1.

Histocytopathology

Peripheral blood smears were performed using 10 µL whole blood at the time of necropsy, and prepared with Wright-Giemsa stain (Fisher; Hampton, NH).

Statistics

SigmaStat was used for all statistical analyses (Systat Software; Point Richmond, CA). Normally distributed data were compared using Student *t*-test. Measurements that were not normally distributed were compared by the nonparametric Mann-Whitney rank sum test. P<0.05 was considered statistically significant. Correlation measurements were obtained by Pearson Correlation.

RESULTS

Mice with melanoma develop anemia with iron-restricted erythropoiesis and inflammation.

We characterized a mouse model of melanoma by injecting a melanoma cell line, B16-F10, into the peritoneum of mice. Control mice received PBS. The melanoma and PBS mice were sacrificed 14 days after the date of injection. At this time, the tumor mice displayed signs consistent with systemic illness and tumor load, including lethargy and ascites. Whole blood complete blood count analysis revealed a marked anemia in the melanoma mice as compared to controls (Fig. 3-1A) (B16-F10 Hgb 8.2 vs. PBS Hgb 15.6, $P<0.001$). The tumor mice also developed a robust reticulocytosis ~2.5x that of their control counterparts (Fig. 3-1B).

When insufficient iron is available for erythropoiesis, increased levels of zinc are incorporated into the protoporphyrin ring. Therefore, zinc protoporphyrin (ZPP) levels are a good indicator of iron-restricted anemia [26]. Our melanoma mice developed significantly elevated ZPP levels as compared to controls (Fig. 3-1C).

In order to evaluate the contribution of inflammation and hepcidin activity to the development of anemia in our cancer models, we measured levels of SAA-1 and hepcidin. SAA-1 is a liver acute phase reactant that is known to be expressed in response to inflammation and certain cytokines, particularly IL-6 [27]. While most of the literature describing the role of IL-6 in stimulating SAA-1 expression is from in vivo and in vitro human specimens, we have data [unpublished] that shows that IL-6 knockout mice have lower SAA-1 levels than wild type mice, and that their SAA-1 levels have a blunted response to inflammation. On day 14, the melanoma mice had a dramatic increase (>600-fold) in SAA-1 mRNA as compared to controls, indicating robust

inflammation (Fig. 3-1D). Surprisingly, hepcidin mRNA levels were suppressed in the tumor mice as compared to controls (Fig. 3-1E).

Mice with metastatic lung cancers develop anemia with reticulocytosis.

We created two models of metastatic lung cancer by injecting two syngeneic cancer cell lines, TC-1 and LLC, into the peritoneum of mice. Control mice received PBS. The TC-1, LLC, and PBS mice were sacrificed 14 days after the date of injection. At this time, all the tumor mice displayed signs consistent with systemic illness secondary to tumor load, including lethargy, poor grooming, and ascites. Necropsy revealed obvious intraperitoneal masses in most of the mice. Whole blood complete blood count analysis revealed a lower hemoglobin in the tumor mice as compared to controls (Fig. 3-2A) (TC-1 vs. PBS, $P=0.029$; LLC vs. PBS, $P<0.001$). The anemic tumor mice had increased reticulocytosis as compared to the controls (Fig. 3-2B) (LLC vs. PBS, $P<0.001$; TC-1 vs. PBC, $P<0.001$). Of note, the LLC mice had lower hemoglobin levels than TC-1 mice ($P<0.001$) and greater reticulocytosis ($P=0.006$).

Mice with metastatic lung cancers have iron-restricted anemia with hypoferremia but increased total body iron stores.

Our mouse tumor models had elevated ZPP levels as compared to controls, indicating iron-restricted erythropoiesis (Fig. 3-3A) ($P<0.001$). LLC mice had a trend towards higher ZPP levels than the less anemic TC-1 mice (LLC vs. TC-1, $P=0.129$). Hypoferremia in the tumor mice was confirmed with a serum iron assay (Fig. 3-3B) (TC-1 vs. PBS, $P=0.031$; LLC vs. PBS, $P=0.004$). In order to determine whether this was due to iron sequestration, as would be seen in anemia of inflammation, rather than iron-deficiency anemia secondary to hemolysis, malnutrition, or bleeding, total body iron stores were evaluated using liver and spleen tissue iron measurements.

Both lung tumor models had higher liver iron content than controls (Fig. 3-3C) (LLC vs. PBS, P<0.001; TC-1 vs. PBS, P=0.002). LLC mice also had higher spleen iron content than controls, and TC-1 mice had comparable spleen iron content (Fig. 3-3D) (LLC vs. PBS, P=0.033; TC-1 vs. PBS, P=0.381).

Mice with metastatic lung cancers have increased inflammation, but varying levels of hepcidin.

At day 14, both TC-1 and LLC mice had elevated liver SAA-1 mRNA levels as compared to controls (Fig. 3-4A) (LLC vs. PBS, P=0.001; TC-1 vs. PBS, P=0.002). As IL-6 is known to be an important cause of the anemia of inflammation by inducing hepcidin expression [19], SAA-1 measurements help to determine the role of inflammation in the development of anemia of cancer. While our lung tumor models did show increased SAA-1 levels, our data showed no clear correlation between the level of inflammation and corresponding hepcidin mRNA levels [data not shown]. The tumor mice also had higher SAA-1 levels than the controls, for comparable hepcidin levels. This difference indicates the presence of another factor(s) suppressing hepcidin synthesis, and these factors could include anemia and increased erythropoiesis.

At day 14, TC-1 mice had elevated liver hepcidin mRNA levels as compared to controls, while LLC mice had lower hepcidin levels (Fig. 3-4B) (TC-1 vs. PBS, P=0.012; LLC vs. PBS, P=0.022). The increased hepcidin production despite the presence of anemia in TC-1 mice suggests an active role of hepcidin in the development of anemia in this model. Tumor hepcidin levels were measured to determine whether tumor hepcidin expression may be contributing to the development of anemia of cancer. Neither TC-1 nor LLC tumors had significant hepcidin expression [data not shown] (TC-1 liver hepcidin vs. TC-1 tumor hepcidin, P<0.001; LLC liver

hepcidin vs. LLC tumor hepcidin, P<0.001), illustrating that these tumors do not directly contribute to systemic hepcidin levels.

T*he ovarian tumor model develops anemia of cancer with iron-deficiency.*

In contrast, we also studied a slow-growing model of anemia of cancer by injecting the syngeneic ovarian cancer cell line ID8 into the peritoneum of mice. Control mice received PBS. The ID8 and PBS mice were sacrificed 18.5 weeks after the date of injection with tumor or PBS. At this time, all the tumor mice displayed signs consistent with systemic illness secondary to tumor load, including lethargy, poor grooming, and ascites. Necropsy revealed obvious intraperitoneal masses in the majority of the mice with large hemoperitoneum noted in some. Whole blood complete blood count analysis revealed a lower hemoglobin in the tumor mice as compared to controls (Fig. 3-5A) (ID8 vs. PBS, P=0.049). The ovarian tumor mice had elevated ZPP levels as compared to controls, indicating iron-restricted erythropoiesis (Fig. 3-5B) (P=0.026). Total body iron stores were evaluated using liver and spleen tissue iron measurements. The ID8 mice had lower liver and spleen iron content than controls (Fig. 3-5C,D) (ID8 vs. PBS liver iron, P=0.037; ID8 vs. PBS spleen iron, P<0.001). This ovarian tumor model develops anemia with frank iron deficiency, likely secondary to hemoperitoneum and nutritional deficiencies.

The ovarian tumor mice have suppressed hepcidin levels, with positive correlation with liver iron stores.

At 18.5 weeks, ID8 mice had depressed liver hepcidin mRNA levels as compared to controls (Fig. 3-6A) (P=0.041), as well as comparable SAA-1 levels to controls (Supplemental Figure 3-1) (P=0.219), pointing away from inflammation and hepcidin as mediators in the development of

this anemia. In addition, figure 3-6B shows a significant positive correlation between hepcidin and hemoglobin levels, which indicates appropriate hepcidin suppression with the development of iron-deficiency anemia in this ovarian tumor model (ID8 hepcidin vs. hemoglobin, R^2=0.64, P=0.0167).

The development of anemia in these three tumor models is not secondary to hemolysis.

The list of potential causes of anemia in cancer models includes hemolysis or shearing of erythrocytes, which can also be seen in inflammatory anemias [6]. The peripheral blood smears of the tumor mice show no evidence of overt microangiopathic hemolysis contributing to their anemia. Figure 3-8B shows a nearly normal blood smear of the TC-1 model, with minimal membrane irregularities. Figures 3-8C and 3-8D show that the more anemic LLC and ID8 models do have more severe erythrocyte abnormalities, including teardrop cells and central pallor, but again without a significant number of schistocytes that would indicate a contribution of microangiopathic hemolysis to anemia. [28]

The fall in hemoglobin with TC-1 or ID8 is not attenuated in hepcidin knockout mice.

In order to test whether hepcidin is necessary for the development of anemia in our TC-1 mice, we repeated the TC-1 treatment in hepcidin knockout mice. At day 14, there was no significant difference between the hemoglobin decreases of wild-type and hepcidin knockout mice treated with TC-1 (Fig. 3-7A) (P=0.908). While TC-1 mice did have inappropriately elevated levels of hepcidin that may contribute weakly to their anemia, hepcidin is clearly not essential for the development of this anemia. Taken together with the increase in SAA-1 levels, these data point to a hepcidin-independent inflammatory mechanism for anemia.

In order to confirm that hepcidin was not an important mediator of the development of anemia in our ovarian tumor model, we repeated the ID8 treatment in hepcidin knockout mice. At 18.5 weeks, there was no significant difference between the hemoglobin decreases of wild-type and hepcidin knockout mice treated with ID8 (Fig. 3-7C) (P=0.739). This data aligns with the above conjecture that these ID8 mice develop anemia in response to bleeding and iron deficiency, rather than a hepcidin-mediated inflammatory mechanism.

DISCUSSION

The anemia of cancer (AC) is an often overlooked but very important aspect of a malignancy diagnosis. Even mild anemia has been shown to adversely affect patient-reported quality of life (QOL) parameters [29]. And although a decreased hemoglobin is only one factor in the development of depressed QOL measurements in cancer patients, the improvement of anemia has been shown to have a significant and direct correlation with the overall QOL [30]. Interestingly, this same study showed that this improvement in QOL with anemia treatment persisted regardless of partial or complete response to any chemotherapy regimen. Additionally, AC has been shown to be an independent negative prognostic factor in survival time for cancer patients [31].

Despite the vital role of anemia in the treatment of cancer, AC is widely believed to be under-recognized and under-treated by physicians [32]. One likely etiology for this undertreatment is the lack of safe and effective therapies. Red blood cell transfusions are associated with immunosuppressive effects, infection transmission, and transfusion reactions [33]. Erythropoiesis-stimulating agents, such as recombinant human erythropoietin, have been associated with higher mortality rates in patients with head and neck cancer, breast cancer, and non-small cell lung cancer [34]. In this study, we have described four different mouse models of AC in order to characterize the development of anemia in various cancers, and to investigate the potential role of hepcidin.

Our melanoma model developed a robust and rapid anemia, with a hemoglobin that dropped to nearly 50% that of controls by 14 days. This anemia was accompanied by an increase in RPI and ZPP, indicating a marked iron-restricted erythropoiesis. As these hematologic findings are

comparable to those found in AI, measurements were also made of inflammatory and hepcidin parameters. While there was a dramatic >600-fold increase in SAA-1, an inflammatory marker, there was also a significant drop in hepcidin mRNA measurements. While hepcidin is increased in times of inflammation, it is also decreased during intensified erythropoiesis [35], so the drop in hepcidin levels in our melanoma model could still represent inappropriately elevated hepcidin production that could contribute to iron restriction.

Our two rapidly-growing mouse models of lung cancer developed anemia of varying severity. The LLC mice had hemoglobins that fell to nearly half that of controls by 14 days, but the TC-1 mice had a milder but still significant anemia. Accordingly, the LLC mice had a robust iron-restricted erythropoiesis, while the TC-1 mice had a milder version of the same pattern. In keeping with the characteristic findings in AI, both lung cancer models had intact (and even increased) tissue iron stores despite the presence of hypoferremia and inadequate circulating iron for red blood cell production. Both lung cancer models had elevated inflammatory markers, but only TC-1 mice had an increase in hepcidin production. This elevation in hepcidin mRNA levels despite the suppressive effect of erythropoiesis pointed to a possible role in hepcidin in the development of anemia in TC-1 mice.

Our mouse model of ovarian cancer was a slow-growing model, with euthanasia performed at 18.5 weeks. While these mice also developed a significant anemia with iron-restricted erythropoiesis similar to our other cancer mouse models, the ovarian cancer mice had reduced tissue iron stores. Coupled with the finding of hemoperitoneum at necropsy, the low tissue iron stores in these ovarian cancer mice lead us to believe that bleeding and frank iron-deficiency were the primary etiologies of anemia in these mice. The finding of decreased hepcidin mRNA

production in these mice also pointed away from hepcidin and AI as important contributors in this model of AC.

In order to investigate the potential role of hepcidin in the development of AC in these models, we repeated the experiments in hepcidin-1 KO mice using one rapidly-growing model and one slow-growing model of AC. As TC-1 mice were the only group to have elevated hepcidin levels despite the suppressive effect of erythropoiesis, we chose this lung cancer cell line as our rapidly-growing model. Interestingly, these experiments showed no difference in the development of anemia in either the TC-1 mice or the ovarian cancer mice. While this does not eliminate the possibility that hepcidin is contributing somewhat to AC in these models, the lack of any perceptible improvement in anemia points to hepcidin-independent factors in anemia development in these mice.

In this paper, we have characterized four different mouse models of AC with both rapidly-growing and slow-growing cell lines. Three of the models developed an anemia that shares some of the characteristics of AI: iron-restricted erythropoiesis, intact tissue iron stores, and inflammation. Despite these findings, the repeated experiments in hepcidin knockout mice did not show any significant contribution of hepcidin in the development of anemia. Our slow-growing mouse model of ovarian cancer developed a frank iron-deficiency anemia, likely secondary to hemorrhagic ascites and long-term anorexia.

Much is yet to be understood about the development of anemia in cancer patients. The characterization and manipulation of mouse models of cancer should be useful for the exploration of mechanisms and potential therapeutics for AC.
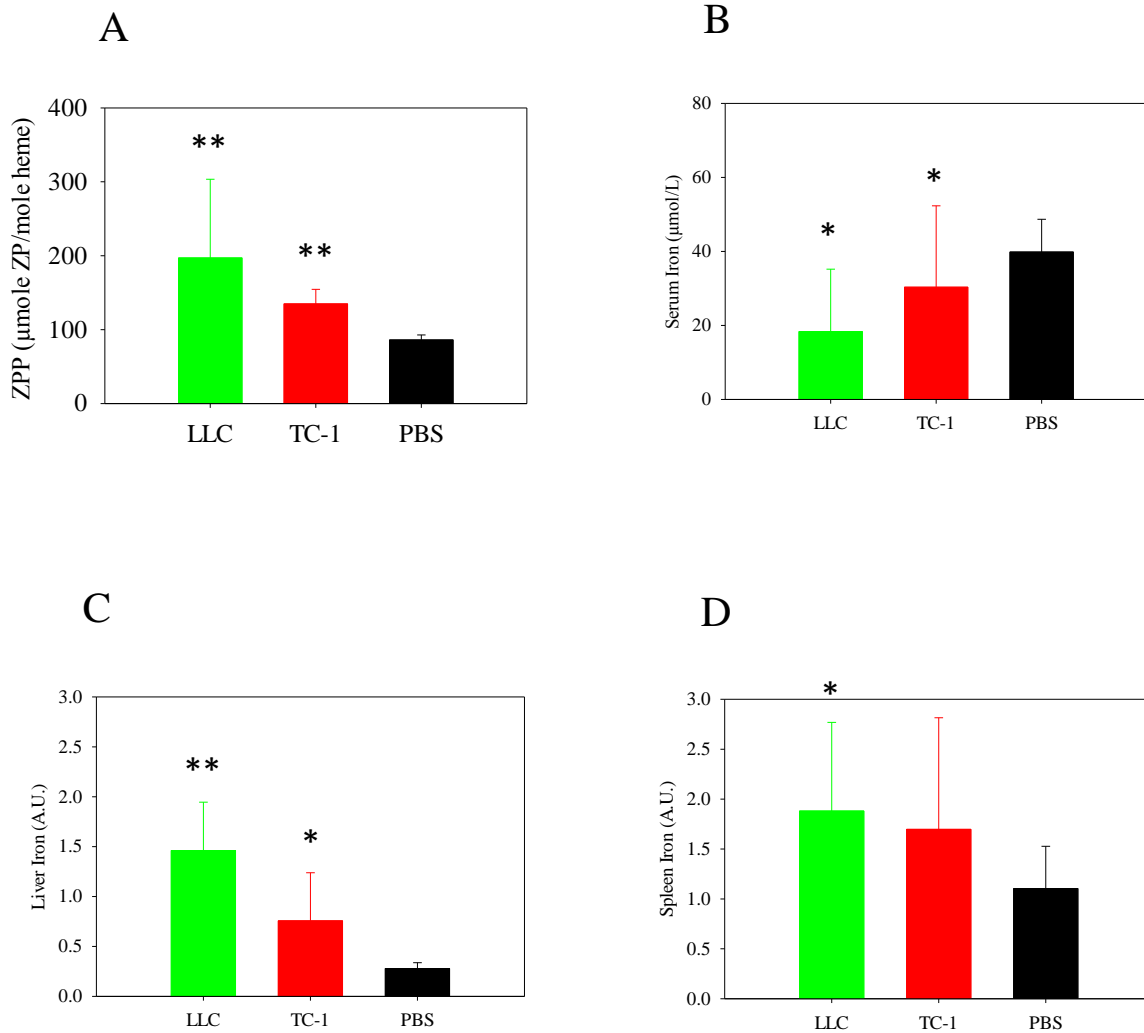
Figure 3-1
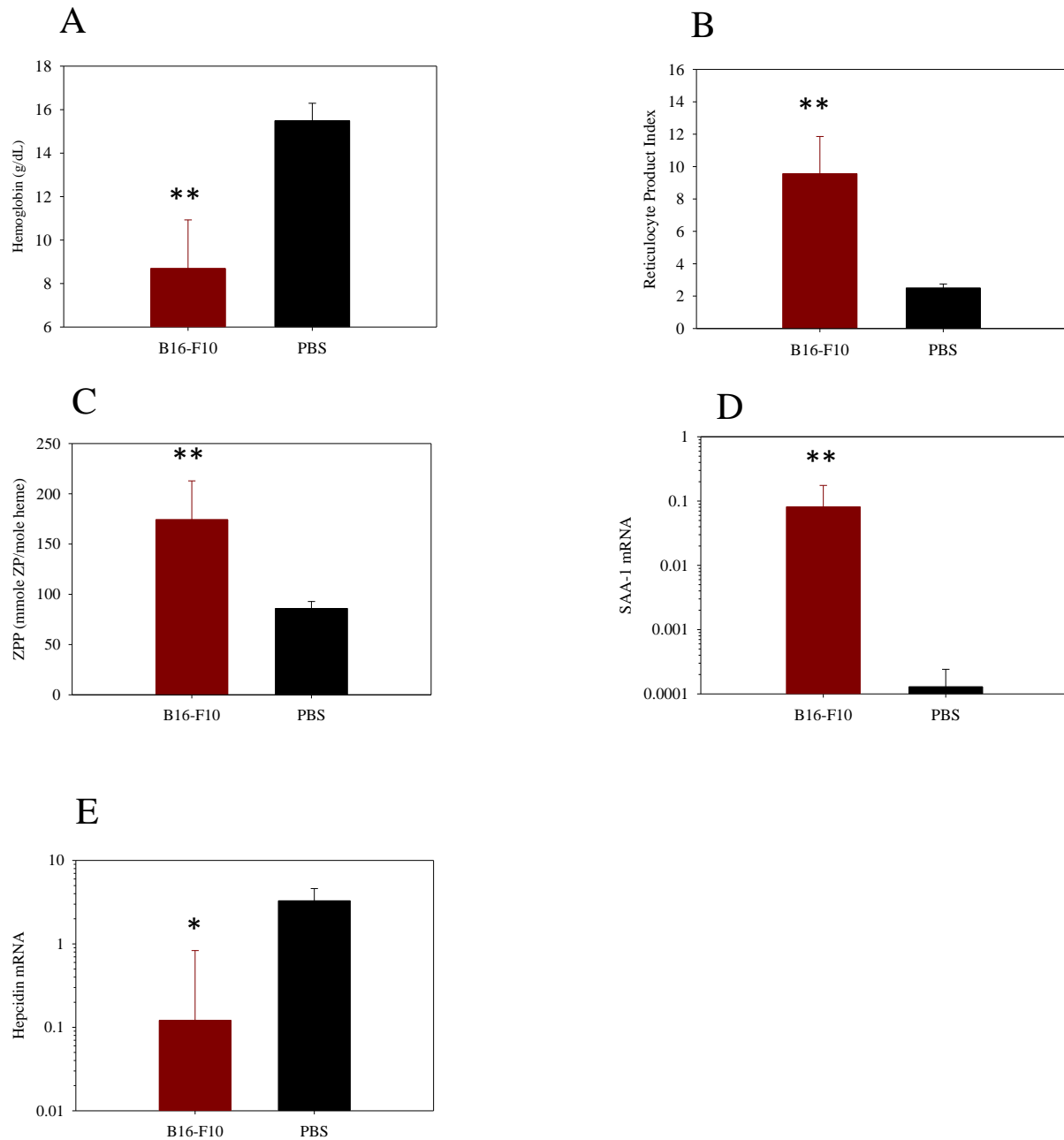
Figure 3-1. The melanoma mice develop anemia with iron-restricted erythropoiesis and inflammation.
C57BL/6 mice were injected intraperitoneally with $0.1x10^6$-$0.3x10^6$ B16-F10 cells, and euthanized after
14 days. (A) The melanoma mice develop a significant anemia. (B) The tumor mice have increased
reticulocytosis as compared to controls. (C) The tumor mice have elevated zinc protoporphyrin (ZPP) levels as
compared to controls, indicating iron-restricted heme synthesis. (D) The melanoma mice have increased levels
of liver SAA-1 mRNA, a marker of inflammation and IL-6 activity. (E) The tumor mice have lower levels of
hepcidin mRNA. *P<0.05, **P<0.001; P by Mann-Whitney rank sum test. Bars and error bars are mean ± SD
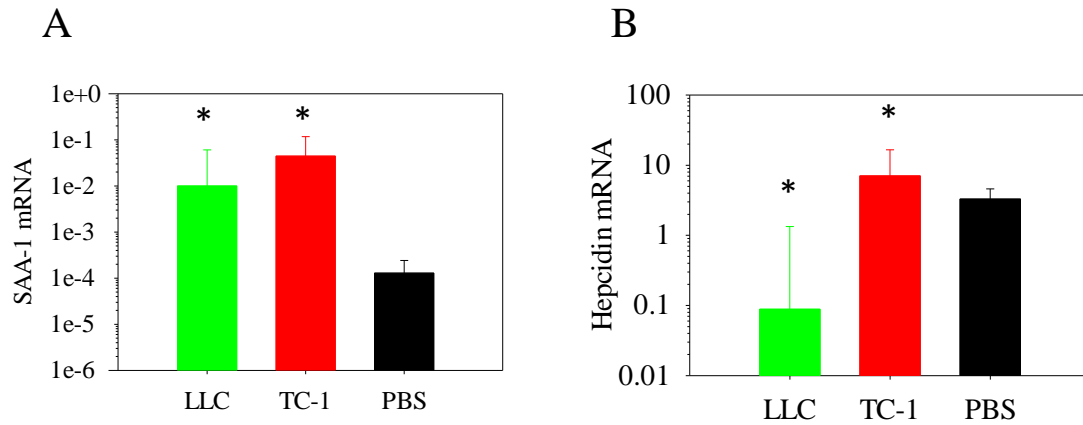(A-C) or median ± 75th percentile/ 25th percentile (D-E).

Figure 3-2
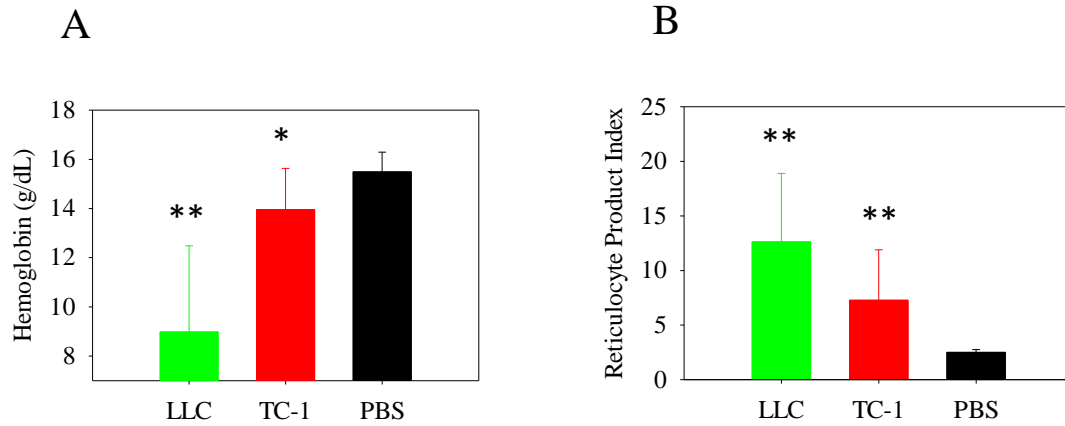
Figure 3-2. The lung tumor mice develop anemia with reticulocytosis. C57BL/6 mice were injected intraperitoneally with 0.1x10^6-0.5x10^6 murine TC-1 or Lewis lung carcinoma (LLC) cells and euthanized after 14 days. (A) The lung tumor mice have a greater drop in hemoglobin as compared to controls. (B) The tumor mice have increased reticulocytosis as compared to controls. *P<0.05, **P<0.001; P by Mann-Whitney rank sum test. Bars and error bars are mean ± SD.
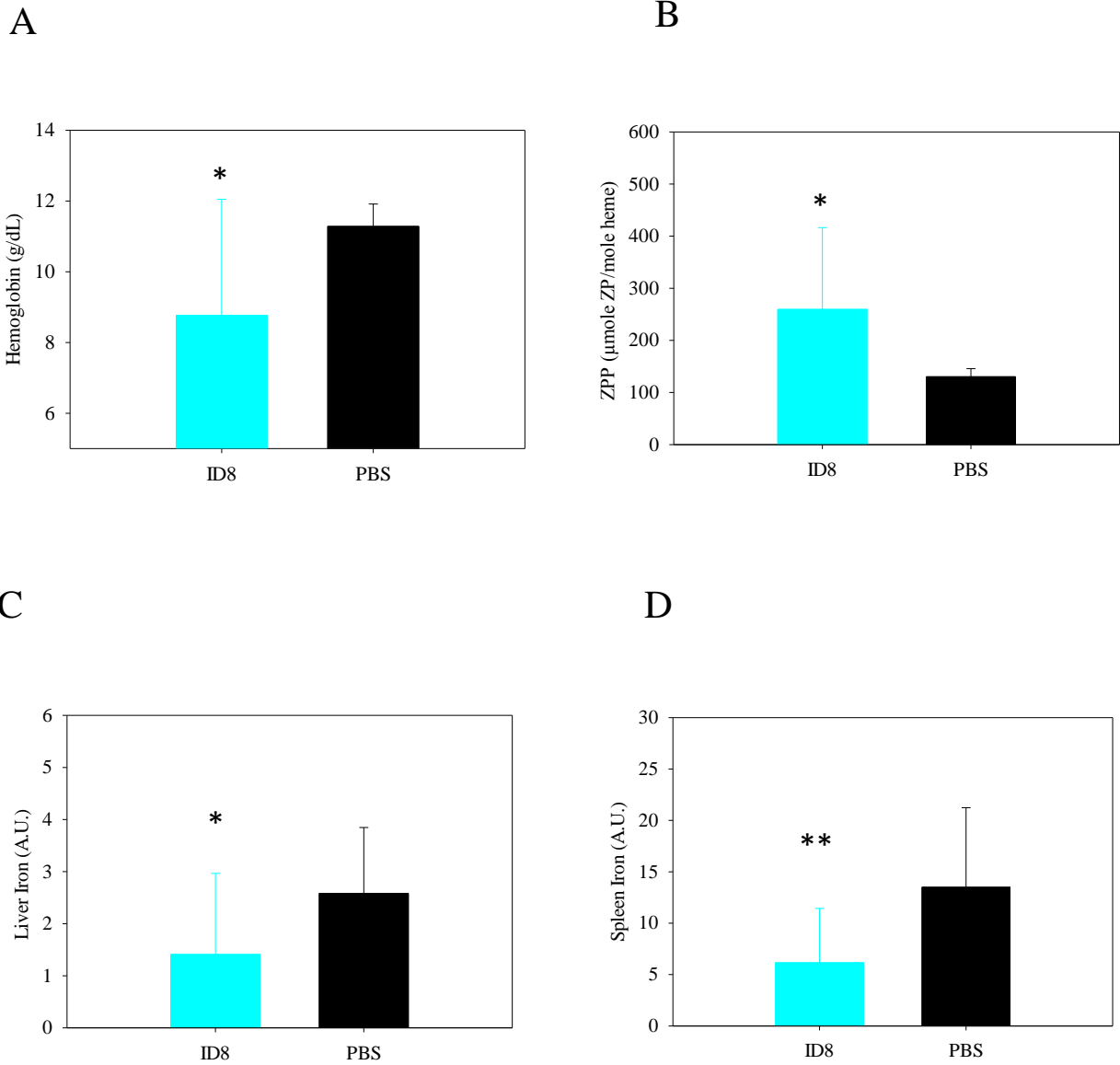
Figure 3-3

Figure 3-3. The lung tumor mice have iron-restricted erythropioesis with hypoferremia, but with intact tissue iron stores. C57BL/6 mice were injected intraperitoneally with TC-1 or LLC cells and euthanized after 14 days. (A) The tumor mice have elevated ZPP levels as compared to controls[a], indicating iron-restricted heme synthesis. LLC mice have a trend towards higher ZPP levels than the less anemic TC-1 mice (LLC vs. TC-1, P=0.129[a]). (B) The tumor mice have relative hypoferremia[a]. (C) Both tumor models have increased liver iron[a]. (D) The LLC mice have increased spleen iron[b], and the TC-1 tumor mice have a trend towards increased spleen iron[a]; TC-1 vs. PBS, P=0.381. *P<0.05, **P<0.001; [a]P by Mann-Whitney rank sum test, [b]P by t-test. Bars and error bars are mean ± SD.

Figure 3-4

A

B

Figure 3-4. TC-1 and LLC mice both have increased SAA-1 mRNA, but only TC-1 has increased hepcidin mRNA. (A) At day 14, both tumor models have increased levels of liver SAA-1 mRNA. (B) By day 14, TC-1 mice have increased liver hepcidin mRNA levels as compared to controls, and LLC mice have decreased hepcidin. *P<0.05; P by Mann-Whitney rank sum test. Bars and error bars are median ± 75th percentile/ 25th percentile.
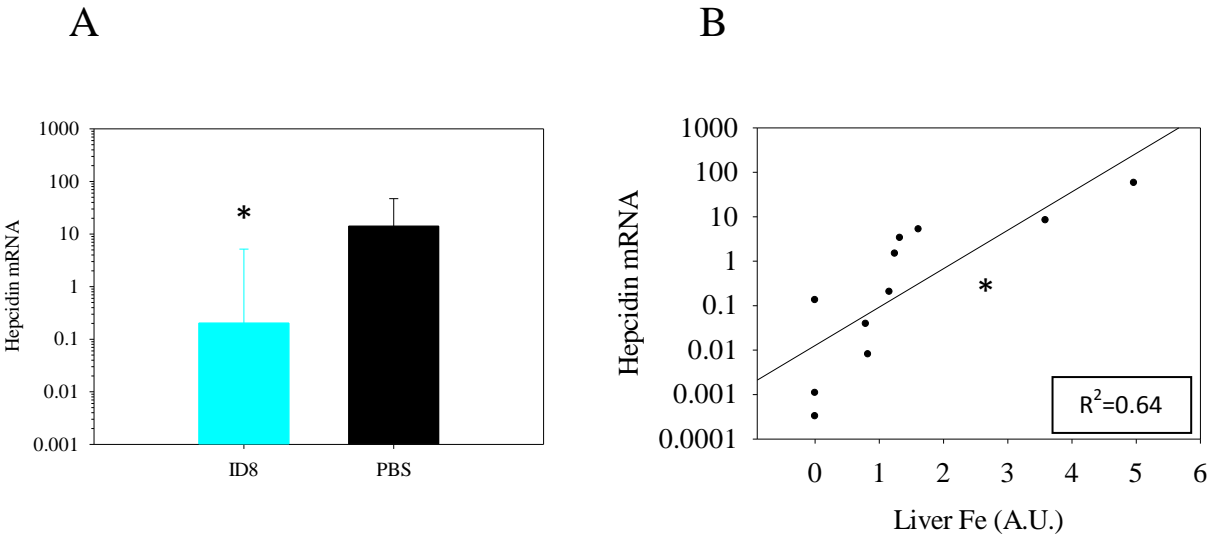
Figure 3-5

A

B

C

D

Figure 3-5. ID8 mice develop anemia with iron-deficiency. C57BL/6 mice were injected intraperitoneally with 1x10^{6} ID8 cells and euthanized after 18.5 weeks. (A) The ID8 mice are more anemic than controls. (B) The ID8 mice have elevated ZPP levels. (C,D) The ID8 mice have lower liver and spleen iron levels, as compared to controls. *P<0.05, **P<0.001; P by Two Way

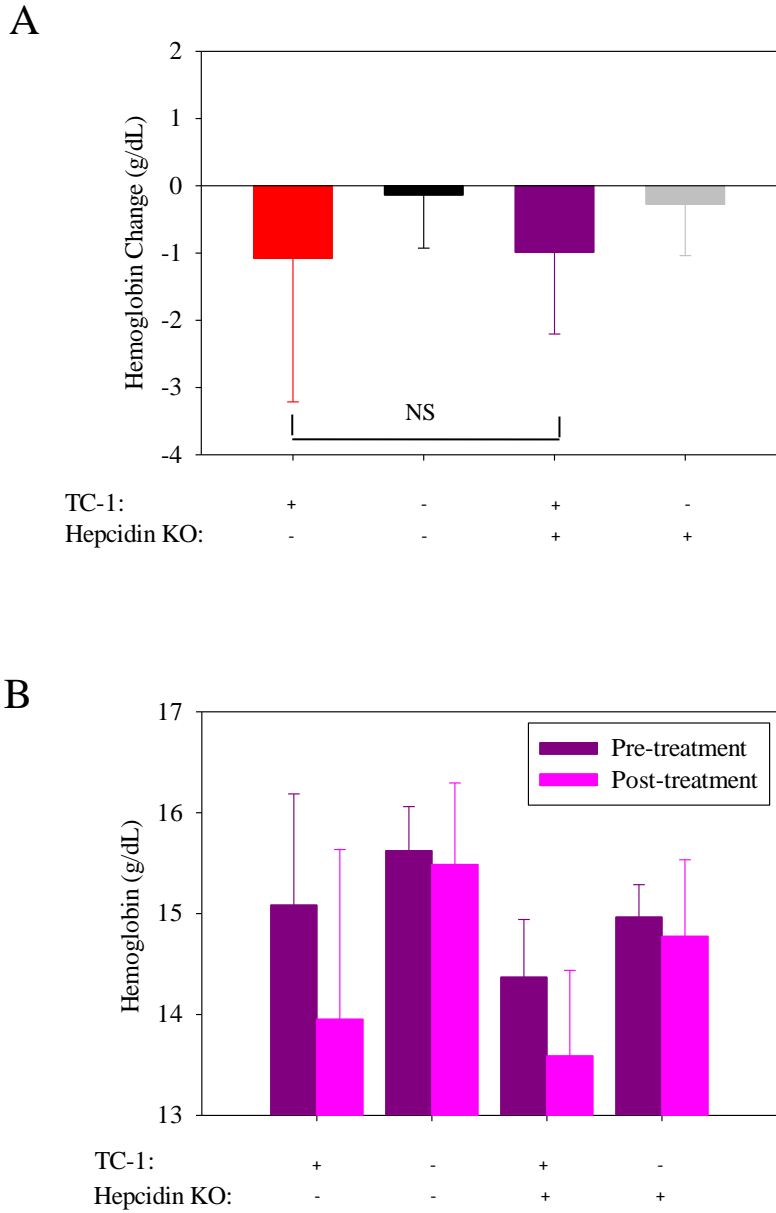Figure 3-6

A

B

Figure 3-6. ID8 mice have decreased hepcidin expression with positive correlation with iron stores. (A) At 18.5 weeks, ID8 mice have suppressed hepcidin expression as compared to controls[a]. (B) ID8 mice have significant correlation between liver iron stores and hepcidin mRNA expression[b]. *P<0.05; [a]P by Two Way ANOVA, [b]P by Pearson Correlation. Bars and error bars are median ± 75th percentile/ 25th percentile.

Figure 3-7A-B

A

TC-1:   +   -   +   -
Hepcidin KO: -   -   +   +

B

TC-1:   +   -   +   -
Hepcidin KO: -   -   +   +
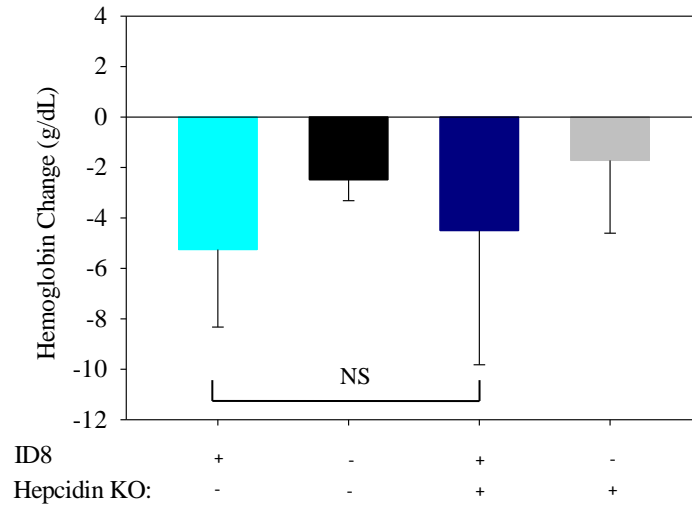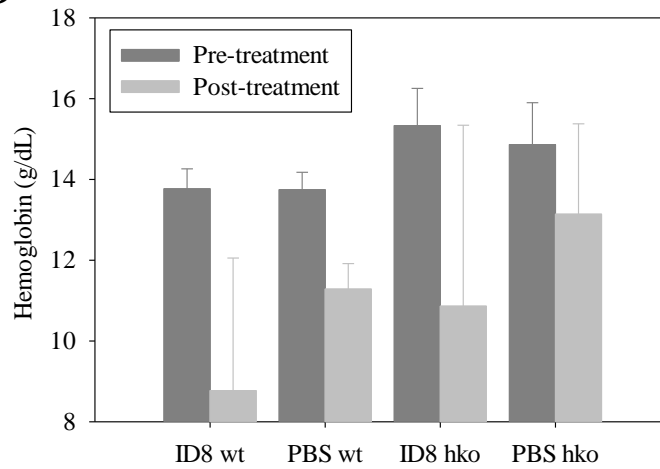
Figure 3-7A,B. The hemoglobin drop with TC-1 treatment is not attenuated in hepcidin knockout mice.
(A) By day 14, there is no significant difference between the hemoglobin changes of wild-type and hepcidin
knockout mice treated with TC-1 (P=0.908). (B) Both the wild-type and hepcidin knockout mice develop
anemia after treatment with TC-1. P by t-test. Bars and error bars are mean ± SD.

Figure 3-7C-D

C

D


Figure 3-7C,D. The hemoglobin drop with ID8 treatment is not attenuated in hepcidin knockout mice. (C) By 18.5 weeks, there is no significant difference between the hemoglobin changes of wild-type and hepcidin knockout mice treated with ID8 (P=0.739). (D) Both the wild-type and hepcidin knockout mice develop anemia after treatment with ID8. P by t-test. Bars and error bars are mean ± SD.
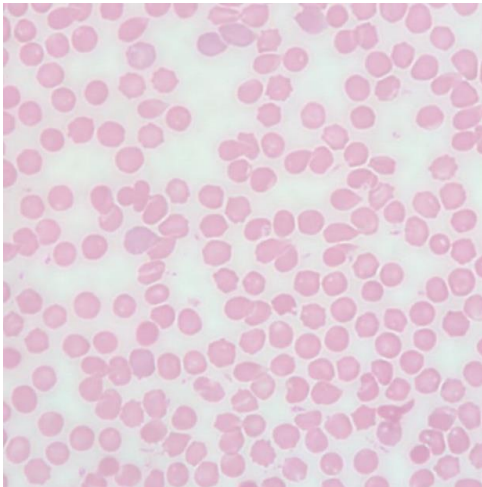
Figure 3-8
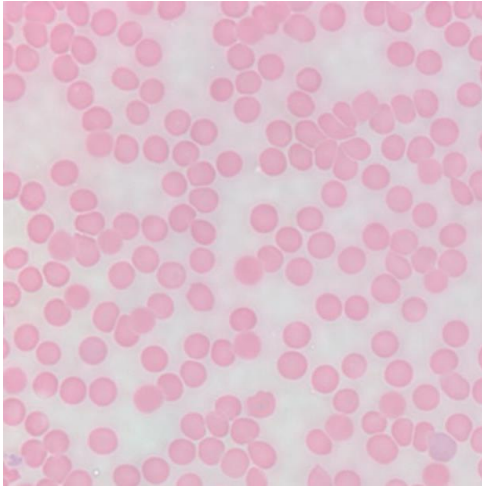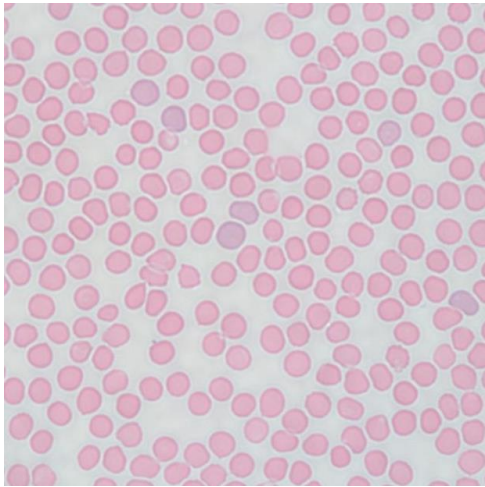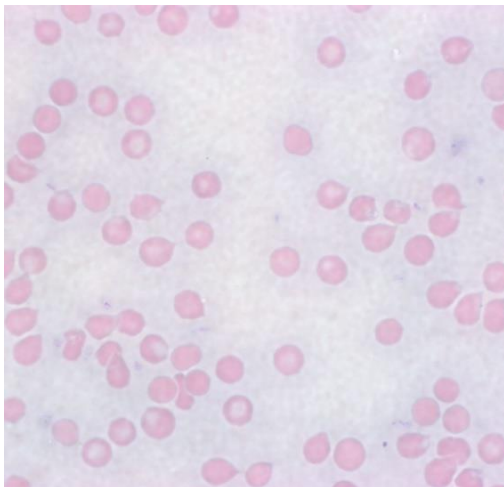
Figure 3-8. Peripheral blood smears from tumor and control mice show no significant schistocytosis.
(A) PBS control (B) TC-1 (C) LLC (D) ID8 in hepcidin knockout mice.

Supplemental Figure 3-1
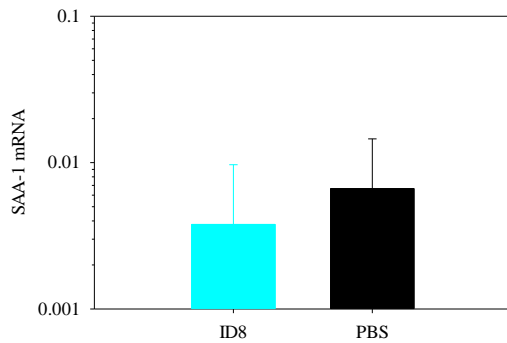
Supplementary Figure 3-1. Ovarian tumor mice SAA-1 expression. By 18.5 weeks, the
ID8 mice and control mice have comparable SAA-1 levels (P=0.219).

Supplemental Table 3-1: PCR Primers

Gene target	Forward Primer	Reverse Primer
Murine HAMP1	5'-CCTATCTCCATCAACAGATG-3'	5'-AACAGATACCACACTGGGAA-3'
Murine β-Actin	5'-ACCCACACTGTGCCCATCTA-3'	5'-CACGCTCGGTCAGGATCTTC-3'
Murine SAA-1	5'-TGACCAGGAAGCCAACAG-3'	5'-GTAGGAAGACCAGACC-3'

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Chapter 4

A MOUSE MODEL OF ANEMIA OF INFLAMMATION: COMPLEX PATHOGENESIS WITH PARTIAL DEPENDENCE ON HEPCIDIN

ABSTRACT

Anemia is a common complication of infections and inflammatory diseases, but the few mouse models of this condition are not well characterized. We analyzed in detail the pathogenesis of anemia induced by an injection of heat-killed *Brucella abortus* (BA), and examined the contribution of hepcidin by comparing wild-type (WT) to iron-depleted hepcidin-1 KO (*Hamp*-KO) mice. BA-treated WT mice developed severe anemia with a Hb nadir at 14 d and partial recovery by 28 d. After an early increase in inflammatory markers and hepcidin, WT mice manifested hypoferremia, despite iron accumulation in the liver. Erythropoiesis was suppressed between days 1 and 7, and erythrocyte destruction was increased as evidenced by schistocytes on blood smears and shortened RBC lifespan. Erythropoietic recovery began after 14 d but was iron-restricted. In BA-treated *Hamp*-KO compared to WT mice, anemia was milder, not iron-restricted and with faster recovery. Similarly to severe human anemia of inflammation, the BA model shows multifactorial pathogenesis of inflammatory anemia including iron restriction from increased hepcidin, transient suppression of erythropoiesis and shortened erythrocyte lifespan. Ablation of hepcidin relieves iron restriction and improves the anemia.

INTRODUCTION

Anemia of inflammation (AI) is a feature of a wide spectrum of inflammatory disorders, including connective tissue disease, infections, certain malignancies, and chronic kidney disease[1]. AI is typically a normocytic normochromic anemia with a shortened erythrocyte lifespan and suppressed erythropoiesis, despite adequate levels of circulating erythropoietin[2]. Perhaps the most consistent feature of AI is a derangement of systemic iron homeostasis characterized by hypoferremia with intact iron stores[1] and decreased availability of iron for erythrocyte production.

Hepcidin, a 25-amino acid peptide hormone produced primarily by hepatocytes[3], is the principal regulator of iron homeostasis in health and during inflammation[4]. Excessive production of this hormone causes iron sequestration in macrophages and hypoferremia, as was shown in transgenic mice with hepcidin overexpression[5] and in the human genetic syndrome of hepcidin excess, iron-refractory iron-deficiency anemia due to mutations in matriptase-2/TMPRSS6[6] . Hepcidin acts by binding to ferroportin, the sole known cellular iron exporter, displayed on the surface of macrophages, hepatocytes, and the basolateral membranes of enterocytes. Hepcidin binding to ferroportin causes ferroportin endocytosis and degradation[7]. During inflammation or infection, hepcidin is strongly induced, largely by IL-6[8] via the JAK-STAT pathway[9-11]. The extent to which increased production of hepcidin contributes to anemia of inflammation has not been directly tested by genetic ablation of hepcidin, in part because of the lack of robust mouse models of AI.

Multiple mouse models of inflammation have been developed to facilitate the testing of interventions in AI, but these models are limited by poor reproducibility and very mild anemia.

Turpentine[12, 13], zymosan/lipopolysaccharide (LPS)[14], *Staphylococcus epidermidis*[15], and complete Freund's adjuvant[15] treatments all cause AI in mice but with a mild anemia (Hgb ≥ 11). LPS injections alone have been shown to cause an acute inflammatory response with hepcidin elevation, but the hematologic effects have not been fully elucidated[16]. Cecal ligation and puncture (CLP) is another potential method for studying AI, but its technical difficulty, variable outcomes, and associated morbidity make it cumbersome for routine use.

In response to the need for a robust mouse model of AI, we have adapted and characterized a model of AI previously described by Sasu, et al.[17], generated with a single intraperitoneal injection of heat-killed *Brucella abortus* (BA). After a comprehensive examination of hematologic, iron, and inflammatory parameters of this model, we investigated the extent of hepcidin involvement in BA-induced AI by comparing wild-type C57BL/6 mice to hepcidin-1^{-/-} mice on the same strain background.

METHODS AND MATERIALS

Animal model of anemia of inflammation

All animal studies were approved by the Animal Research Committee at University of California, Los Angeles (UCLA). Only male mice were used in the study to avoid the effects of gender-related differences in iron parameters and hepcidin [18]. C57BL/6J mice were obtained from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratories (Bar Harbor, ME). Wild-type (WT) mice were maintained on a standard chow (approx. 270 ppm iron, Harlan Teklad, Indianopolis, IN) until 6 weeks of age and they were then switched to an adequate-iron diet (50 ppm iron, Harlan Teklad) for 2 weeks before injection of BA or saline. The same iron-adequate diet was used through the remainder of the experiment. The dietary conditioning was applied because high iron content of standard chow maximally stimulates hepcidin expression and renders it unresponsive to inflammatory stimuli [8], and because dietary iron absorption in humans accounts for ~5-10% of the daily iron fluxes but as much as ~50% in mice fed standard chow [19]. High dietary iron absorption in mice may obscure the contribution of iron recycling by macrophages [20] and leads to progressive iron loading. Reducing the dietary iron content of mouse chow was designed to model iron fluxes of human homeostasis.

To induce anemia of inflammation, animals were injected intraperitoneally with 5×10^8 particles/mouse of heat-killed *Brucella abortus* (BA; strain 1119-3; US Department of Agriculture, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories) as previously described[17]. Control mice were injected intreperitoneally with normal saline (NS). Mice were euthanized over a time-course (0-28 days) and blood, liver, spleen, pancreas and kidney were collected at necropsy. For each time point, 8 saline and 8 BA-

injected mice came from the same cohort and were processed simultaneously to minimize cohort-to-cohort variability, yielding 4-8 evaluable samples per group per time point.

For the study of the role of hepcidin in anemia of inflammation, we used male hepcidin-1 knockout mice (*Hamp1*^{-/-} or *Hamp*-KO). *Hamp*-KO mice were originally provided to our laboratory by Dr. Sophie Vaulont[21] and were backcrossed onto the C57BL/6 background as previously described[22] using marker-assisted accelerated backcrossing. For this study, *Hamp*-KO mice underwent dietary iron conditioning to prevent the development of iron overload and maintain iron levels comparable to those of WT mice. At weaning (3-4 wks of age), *Hamp*-KO mice were placed on a low-iron diet (4 ppm) for 2 weeks followed by a 20 ppm iron diet for 2 weeks. This regimen allows for iron depletion without development of anemia. At 7-8 wks of age, the mice were injected with *B. abortus* or saline and switched to an adequate-iron diet (50 ppm) to match the experimental conditions used for WT mice. *Hamp*-KO mice (3-8 evaluable per group and time point) were analyzed before as well as 7, 14, 21 and 28 d after BA or saline injection.

Measurement of iron parameters and erythropoietin

Serum iron and liver non-heme iron concentrations were determined by a colorimetric assay for iron quantification (Sekisui Diagnostics; Lexington, MA) as previously described[19]. Serum erythropoietin was measured by a solid-phase enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D; Minneapolis, MN).

Hematologic studies

Complete blood counts were obtained with a HemaVet blood analyzer (Drew Scientific; Waterbury, CT). To assess iron-restricted erythropoiesis, zinc protoporphyrin (ZPP) was measured using a hematofluorometer (AVIV; Lakewood, NJ).

Reticulocytes were counted by flow cytometry. Blood (5 µl) was added to 1 ml of thiazole orange in phosphate buffered saline with 0.1% sodium azide (PBS-azide, BD Bioscience; San Jose, CA) and incubated at room temperature for 1-3 h. As an unstained control, blood was added to PBS-azide without thiazole orange. The percentage of red-fluorescent reticulocytes (Retic %), was measured by flow cytometry at the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards CA-16042 and AI-28697, and by the JCCC, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA. Unstained controls were used to establish a gate to exclude background fluorescence. The results are expressed as the reticulocyte product index: RPI = Retic % x Hgb / 14.46, where 14.46 g/dL is a mean baseline Hgb level of healthy WT mice.

In vivo erythrocyte biotinylation

WT mice were injected intraperitoneally with saline or BA. On days 5 and 6, the mice were administered NHS-PEG4-Biotin (500 µg, Pierce; Rockford, IL) in PBS by retro-orbital injection. A total of 5 injections were given to maximize biotinylation efficiency. Over the subsequent two weeks, periodic retro-orbital bleeds (25-50 µl) were obtained to quantify the fraction of biotinylated erythrocytes (timeline indicated in Supplemental Figure 4-1). Each sample was divided into two aliquots: one to be labeled with anti-TER-119 (unstained) and the other with anti-TER-119 and streptavidin (stained). The labeling was performed as follows: 5 µL whole

blood was diluted to 500 μL with Hank's Balanced Salt Solution (HBSS) (Invitrogen; Carlsbad, CA) and 1% bovine serum albumin (BSA) (Rockland; Gilbertsville, PA), and 2.5 μL rat anti-mouse TER-119-phycoerythrin added (BD Pharmingen; San Jose, CA). The stained group was also treated with 0.5 μL streptavidin-Alexa 488 (Molecular Probes; Eugene, OR). After incubation at room temperature for 1 h, the samples were centrifuged and resuspended with HBSS/BSA. Flow cytometry was used to determine the % biotinylated erythrocytes (% stained − % unstained).

Serum hepcidin measurement

Mouse serum hepcidin-1 was measured by a sandwich ELISA[23] (courtesy of B. Sasu and K. Cooke, Amgen, Thousand Oaks, CA). The assay was physiologically validated in our laboratory as described in Supplemental Data (Experimental Validation of the Amgen sandwich ELISA for mouse hepcidin-1).

RNA isolation and real-time quantitative PCR

Total RNA was isolated from liver and analyzed by real-time RT-PCR as described previously[24]. Primers are listed in Supplemental Table 4-1.

Histocytopathology

Peripheral blood smears were performed using 10 μL whole blood at the time of necropsy, and prepared with Wright-Giemsa stain (Fisher; Hampton, NH). Formalin-fixed, paraffin-embedded liver and kidney tissues were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) or Masson's trichrome stain at the UCLA Translational Pathology Core Laboratory. Immunoperoxidase staining with 1 μg/ml rat anti-mouse monoclonal antibody to the macrophage

marker F4/80 (AbD Serotec, Kidlington, UK) was performed on deparaffinized sections after unmasking for 3 min with 20 μg/ml proteinase K. Sections were incubated overnight with the anti-F4/80 Mab solution, developed and counterstained with hematoxylin, all using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Statistics

SigmaStat 11 was used for all statistical analyses (Systat Software; Point Richmond, CA). Normally distributed data were compared using Student *t*-test. Measurements that were not normally distributed were compared by the nonparametric Mann-Whitney rank sum test. $P < 0.05$ was considered statistically significant, except for multiple comparisons where the Bonferroni correction was applied.

RESULTS

BA-treated WT mice develop severe anemia with iron restriction

A single treatment with the inflammatory agent BA caused a dramatic hemoglobin decrease by 7 d with a nadir at 14 d (Figure 4-1A, mean BA Hgb 6.8 g/dL vs. saline Hgb 15.0 g/dL; $p < 0.001$ by Mann-Whitney rank sum test). Afterwards, there was a gradual but incomplete hemoglobin recovery by day 28 (BA Hgb 10.7 g/dL vs. saline Hgb 14.7 g/dL; $p < 0.001$ by t-test).

BA-injected mice rapidly developed a prolonged hypoferremia as compared to saline-treated mice (Figure 4-1B). There was a transient increase in serum iron at 3 d, which was attributed to transient erythroid suppression and underutilization of circulating iron, as documented by subsequent data. However, all other time points showed a significant decrease in serum iron ($p < 0.05$ by t-test). Additionally, within the first week, the mean corpuscular volume (MCV) was lower in the BA-treated mice, consistent with iron-restricted anemia[25] (Figure 4-1C). Later MCV measurements (14-28 days) in the BA group were not significantly different from the control group likely because of the increased number of larger stress reticulocytes in BA mice.

Erythrocyte ZPP levels were measured to further assess iron-restricted erythropoiesis (Figure 4-1D). When insufficient iron is available for erythropoiesis, increased amounts of zinc are incorporated into the protoporphyrin ring[26]. The BA-treated mice had comparable ZPP levels to untreated mice until a dramatic increase by 14 d (mean BA ZPP 319 vs. saline ZPP 140; $p < 0.001$ by t-test), with an eventual peak at 21 d (BA ZPP 462 vs. saline ZPP 152; $p < 0.001$ by Mann-Whitney rank sum test) and a partial recovery of BA ZPP by 28 d. As will be seen further on, the ZPP increase coincides with accelerated erythropoiesis during recovery from anemia.

BA-treated WT mice develop acute and chronic inflammation, with an early rise in hepcidin

We assessed the acute phase response to BA by measuring serum amyloid A1 (SAA1) mRNA, which like hepcidin, is regulated by IL-6[27]. BA-treated mice developed an early-onset and persistent SAA1 mRNA elevation starting by 3 h and lasting through 14 d (Figure 4-2A). In parallel with the increase in SAA1, hepcidin mRNA levels (Figure 4-2B) peaked at 6 h with >6-fold increase over their saline counterparts ($p < 0.05$ by t-test), and serum hepcidin protein levels (Figure 4-2C, see Supplemental Data for assay validation) were increased >3-fold at 6 hours ($p < 0.001$ by t-test). Subsequently, hepcidin mRNA levels fell rapidly to levels similar to those of the saline-treated mice, perhaps responding to the developing hypoferremia (Figure 4-1B). However, "normal" hepcidin levels at 7 d are probably inappropriately high [28] considering the profound anemia and very high Epo production in BA mice (shown in Figure 4-3) at that time point. By 14 d, BA-treated mice had significantly decreased hepcidin mRNA (<10% as compared to saline controls; $p < 0.001$ by t-test), presumably secondary to the known suppressive effect of increased erythropoiesis on hepcidin production[28].

The BA-treated mice also developed chronic inflammation. By 7 d, the BA-treated mice developed a dramatic leukocytosis (BA WBC 29.6 K/µL vs. saline WBC 8.2 K/µL; $p < 0.001$ by t-test) which incompletely resolved by day 28 (BA WBC 11.6 K/µL vs. saline WBC 6.7 K/µL; $p < 0.05$ by t-test) (Figure 4-2D). Figure 4-2E demonstrates the hepatic perivascular infiltration of inflammatory cells in BA-treated mice at 7 d which is contemporaneous with the increase in circulating white blood cells. By 14 d, hepatic granulomas were observed containing predominantly F4/80-positive macrophages (Supplemental Figure 4-2A,B).

Suppression and recovery of erythropoietic response in BA-treated mice

By 7 d, serum erythropoietin levels were increased in BA-treated mice (Figure 4-3A) (>12-fold increase over controls; p<0.001 by t-test) consistent with anemia (Figure 4-1A). By 21 d, as the anemia began to resolve, serum erythropoietin of the BA-treated mice started to fall (<8-fold increase over controls; p<0.001 by Mann-Whitney rank sum test).

Reticulocyte measurements showed initial reticulocytosis at 3 h after BA injection (Figure 4-3B) but this is likely due to massive release of stored near-mature reticulocytes rather than new production. During the first week, reticulocyte production decreased in BA mice (Figure 4-3B) indicating erythropoietic suppression. By 14 d, corresponding to the time of the highest erythropoietin levels and lowest hepcidin expression, reticulocytosis increased and peaked at 21 d (>3-fold increase over controls; p<0.001 by Mann-Whitney rank sum test). Because "stress" reticulocytes produced during stress erythropoiesis are significantly larger than mature erythrocytes, an increased percentage of reticulocytes effectively increased the RDW between days 14 and 28 (Figure 4-3C).

Shortened RBC lifespan and increased RBC hemolysis contribute to BA-induced anemia

An RBC biotinylation assay to compare the RBC lifespans of BA-treated and saline control mice (Figure 4-4A) demonstrated a 2.5-fold shortening of the erythrocyte lifespan in BA-treated mice, indicating that increased RBC destruction was contributing to the anemia. Erythrocyte abnormalities in BA-treated mice were most prominent at 14 d (Figure 4-4B), with schistocytes and RBC membrane irregularities indicative of membrane damage. However, the condition was relatively mild, as indicated by schistocytes comprising less than 1% of erythrocytes even at 14 d (see the next section). For comparison, in a human disorder with prominent microangiopathic hemolysis such as the hemolytic-uremic syndrome, the % schistocytes ranged from 2-9%[29]

Microscopic examination of H&E, PAS and Masson trichrome stains of liver, kidney, spleen and pancreas sections obtained at autopsies of BA- and saline-treated mice revealed foci of thrombosis in small arteries and veins in the liver and the kidneys (Supplemental Figure 4-2C,D) as well as in peripancreatic adipose tissue (not shown), indicative of endothelial injury and microthrombosis that could cause microangiopathic hemolysis.

The contribution of hepcidin to BA-induced hypoferremia and anemia

We next examined the development of hypoferremia and anemia in BA-treated hepcidin KO mice (*Hamp*-KO). Prior to use in this study, *Hamp*-KO mice were placed for 4 weeks on an iron-restricted diet to prevent the massive iron overload characteristic of *Hamp*-KO mice, so that they were as similar as possible to WT mice except for the effect of hepcidin during inflammation in the latter. Iron-depleted *Hamp*-KO mice had similar blood hemoglobin as their WT counterparts (Figure 4-5A). Like WT mice, BA-treated *Hamp*-KO mice became inflamed, as indicated by increased SAA-1 mRNA levels and leukocytosis (Supplemental Figure 4-3). Like WT mice, *Hamp*-KO mice showed erythrocyte fragments on blood smears, most prominently at 14 d (0.7% ± 0.5% of erythrocytes in WT, 0.5% ± 0.1% in *Hamp*-KO, n = 4 mice each group, difference not significant by t-test). Both BA-treated groups (*Hamp*-KO and WT) reached their hemoglobin nadir at 14 d, but the anemia of the *Hamp*-KO mice was much milder than in the WT mice (*Hamp*-KO BA Hgb 9.8 g/dL vs. WT BA Hgb 6.8 g/dL; p<0.001 by Mann-Whitney rank sum test). Moreover, the *Hamp*-KO BA-treated mice recovered completely by 28 d, while the WT BA mice did not (*Hamp*-KO BA Hgb 15.1 g/dL vs. WT BA Hgb 10.7 g/dL; p<0.001 by t-test).

As expected, compared to WT mice, serum iron in *Hamp*-KO mice remained elevated even on an iron-restricted diet that substantially depleted their iron stores (Figure 4-5B). Unlike WT mice, the BA-treated *Hamp*-KO mice did not develop hypoferremia but on the contrary, showed increased serum iron. At 14 d, the BA-treated *Hamp*-KO mice had a nearly two-fold increase in serum iron as compared to saline-treated *Hamp*-KO mice ($p < 0.05$ by Mann-Whitney rank sum test). Higher serum iron is likely due to increased destruction of RBCs after BA treatment and recycling of their iron content. ZPP, an index of iron-restricted erythropoiesis, changed very little in BA-treated *Hamp*-KO mice as compared to WT mice (Figure 4-5C).

Compared to the saline *Hamp*-KO group, BA-treated *Hamp*-KO mice transiently decreased their liver iron stores at 7 d and 14 d, indicating that *Hamp*-KO mice mobilized iron from stores in response to anemia. In contrast, despite anemia and increased erythropoietic activity, BA-treated WT mice had increased liver iron stores compared to their saline counterparts suggesting that hepcidin prevented efficient mobilization of iron from the liver (Figure 4-5D).

Interestingly, *Hamp*-KO mice had significantly decreased survival compared to their WT counterparts (Supplemental Figure 4-4). Thus, while hepcidin ablation was protective against the development of severe anemia and iron restriction, hepcidin deficiency or the associated iron redistribution may have impacted BA-induced inflammation or its damaging effects on tissues and organs.

DISCUSSION

Anemia of inflammation (AI, also called anemia of chronic disease) is a mild to moderate, usually normocytic anemia seen in the context of infections and systemic inflammatory disorders including rheumatologic disorders, inflammatory bowel diseases and malignant neoplasms[1]. In addition, chronic kidney diseases often manifest inflammation and associated features of AI. A related condition, anemia of critical illness, develops in ICU settings within days[30, 31]. The pathogenesis of AI is thought to involve a prominent component of inflammation-induced iron restriction as well as impaired proliferation of erythroid precursors, resistance to erythropoietin, and shortened erythrocyte survival[1, 32] and may be further exacerbated by blood loss and iron deficiency. Progress in understanding these and additional mechanisms has been hampered by the lack of a simple and reproducible animal model that can be genetically manipulated.

In this study, we extensively characterized a mouse model of AI induced by heat-killed *Brucella abortus*. In contrast to other mouse models of AI, where anemia was generally very mild, the BA-induced anemia results in a nadir hemoglobin that is <50% of the controls. Remarkably, the BA model is technically simple, requiring only a single injection, and it is robust and reproducible at each time point, with small standard deviations for most hematologic, iron and inflammatory parameters measured. The model has features of both acute and chronic inflammation, as evidenced by the early SAA-1 increase and the delayed leukocytosis and hepatic perivascular infiltration of inflammatory cells.

Clinically, this subacute mouse model of moderately severe AI resembles human AI in severe infections, or in critically ill patients in intensive care units or in patients with severe exacerbations of systemic autoimmune diseases. It remains to be tested whether lower dosing of

heat-killed BA with repeated administration would result in a less severe and more chronic model, with steady state anemia similar to classical anemia of chronic disease.

Our mouse model manifests the key mechanisms implicated in human AI, including hypoferremia and iron-restricted erythropoiesis with preserved iron stores, shortened erythrocyte lifespan and depressed erythropoiesis. Hypoferremia developed rapidly, probably as a consequence of acute increase in hepcidin mRNA and plasma levels driven by inflammation after BA injection. Although following the initial rise hepcidin fell to control levels during the first week, this may not have been sufficiently low in the face of the developing anemia, further contributing to iron restriction. Elevated liver iron in BA-treated WT mice compared to their saline counterparts further supports the role of hepcidin in preventing effective iron mobilization for the recovery from anemia.

The shortened erythrocyte lifespan in the BA mouse model may be a consequence of macrophage activation by cytokines[32, 33] and/or microangiopathic hemolysis. In support of the latter mechanism, arteriolar and venous microthrombosis was observed in multiple tissues and fragmented red cells were apparent on blood smears. However, we did not detect the characteristic deposition of fibrin in renal glomerular capillaries, and schistocytes were relatively rare suggesting that the condition was not severe. Increased erythrophagocytosis by inflammation-activated macrophages was documented in the companion manuscript, and may well be the predominant mechanism shortening the erythrocyte lifespan.

Depressed erythropoiesis is evidenced in our model by a transient suppression of reticulocyte counts despite adequate levels of circulating erythropoietin. The blunted response of the bone marrow to the erythropoietic hormone has been seen in other inflammatory mouse models,

including a turpentine-induced sterile abscess model[12] and a chronic IFN-γ overproduction model. Libregts et al.[33] demonstrated that IFN-γ stimulation promotes monocytic differentiation at the expense of erythroid differentiation, suggesting that erythroid suppression may be a side effect of the increased production of defensive myeloid cells during infection. Interestingly, we observed significantly increased leukocytosis in WT BA as compared to *Hamp*-KO BA mice (Supplemental Figure 4-3B). Following recent suggestions[33, 34], we suspect that the increase in IFN-γ together with hepcidin-mediated iron restriction may promote erythropoietic suppression and a shift to leukocyte production. We speculate that delayed or inadequate shift to leukocyte production in *Hamp*-KO mice may have interfered with the neutralization of BA-associated pathogenic molecules thereby increasing mortality. Although leukocytes are usually thought of as cells that mediate host defense against live microbes, a leukocyte defect that delayed the breakdown of heat-killed Aspergillus hyphae administered to mice worsened inflammatory tissue injury[35].

Importantly, we were able to demonstrate that our mouse model of AI was partially hepcidin-dependent. The ablation of the hepcidin gene resulted in a dramatic reduction of iron-restricted erythropoiesis, and improved mobilization of iron from tissue stores. At the nadir, the hepcidin-1 KO mice had hemoglobin measurements that were 3 g/dL higher than in their WT counterparts, with a faster recovery to normal values. However, the *Brucella*-treated hepcidin-1 KO mice were still markedly anemic at their nadir, with a hemoglobin level 5.8 g/dL below that of controls. While hepcidin appears to play an important role in the development of AI in this model, other mechanisms contribute significantly.

The companion paper by Gardenghi et al. shows strikingly similar findings with the same mouse model of AI. They observed a similar pattern of early hepcidin increases followed by marked

iron-restricted anemia and a slow recovery. Using complementary studies, Gardenghi et al. also demonstrated erythropoietic suppression and shortened erythrocyte lifespan. Flow cytometry confirmed an absence of early erythroid progenitor cells in the bone marrow, and a biotinylation assay showed an increased rate of erythrocyte elimination in the BA-treated mice. Hepcidin-1 KO mice in their experiments also had a milder anemia with an accelerated recovery. Erythrocyte survival in BA-treated hepcidin-1 KO mice was shortened similarly to that of WT mice. Interestingly, Gardenghi et al. found that the ablation of IL-6 also offered a protective effect against AI, albeit weaker than the effect of hepcidin-1 ablation.

As illustrated by its original use[17] and the current studies by us and Gardenghi et al, the *Brucella abortus* model of AI should be useful for the exploration of the mechanisms and possible treatments of AI.
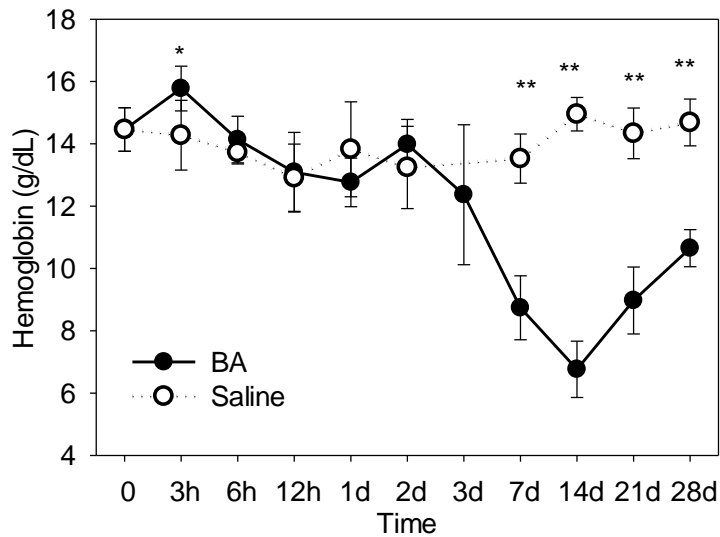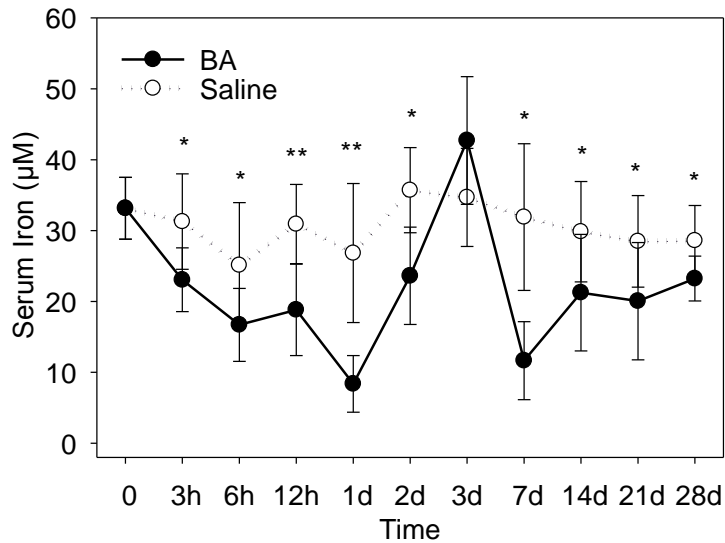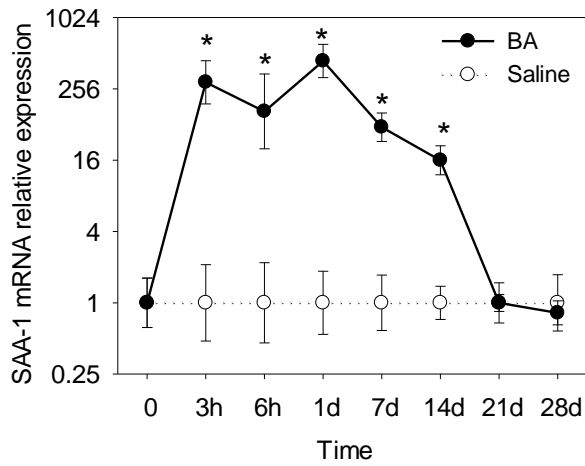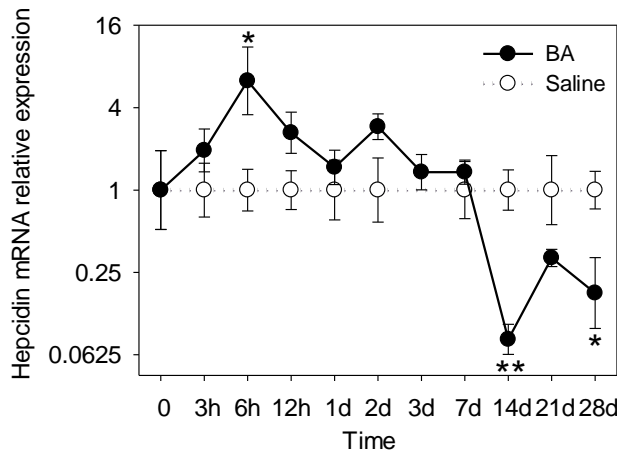
Figure 4-1A,B. The BA-injected mice develop anemia and iron restriction. Compared to saline-injected mice, the BA-injected mice develop: (A) a significantly lower hemoglobin by 7 d, with a nadir at 14 d and partial recovery by 28 d; (B) decreased serum iron concentrations through most time points, beginning at 3 h, except for the transient underutilization of iron around day 3 during erythropoiesis suppression. Saline and BA groups each included 4-8 evaluable male mice per time point. Means ± SD are shown, *p<0.05, **p<0.001; p by t-test or Mann-Whitney rank sum test.
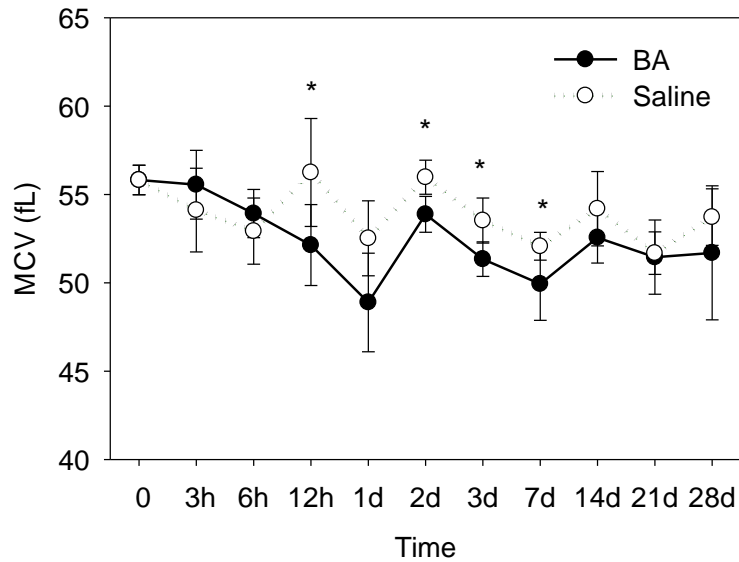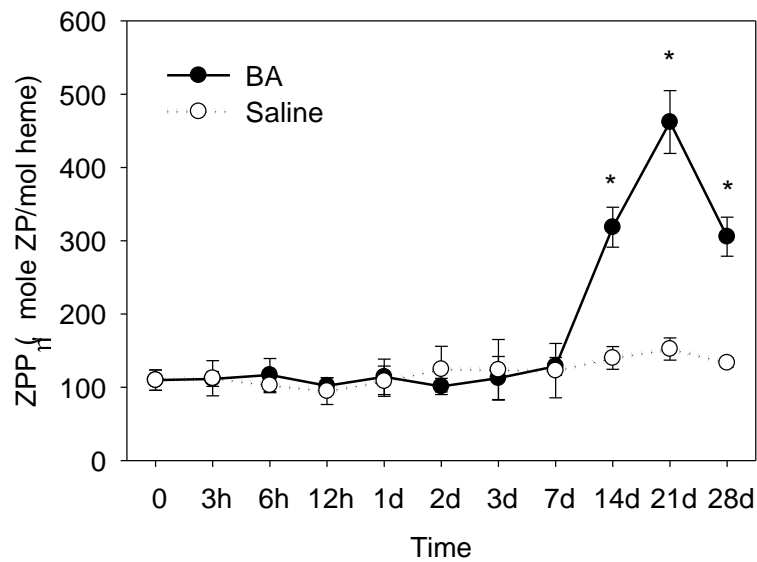
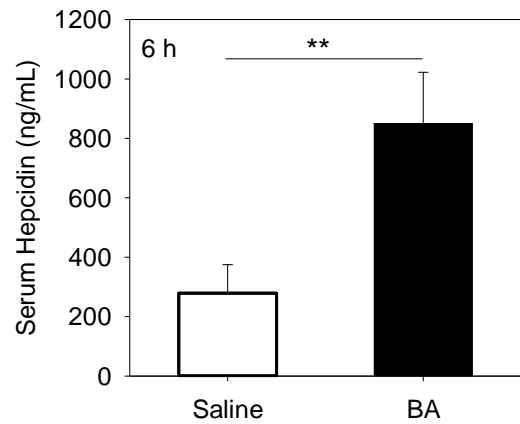**Figure 4-1C,D. The BA-injected mice develop anemia and iron restriction.** Compared to saline-injected mice, the BA-injected mice develop: (C) decreased mean corpuscular volumes within the first week; and (D) elevated zinc protoporphyrin levels between 14 and 28 d during the time of recovery from anemia. Saline and BA groups each included 4-8 evaluable male mice per time point. Means ± SD are shown, *p<0.05, **p<0.001; p by t-test or Mann-Whitney rank sum test.
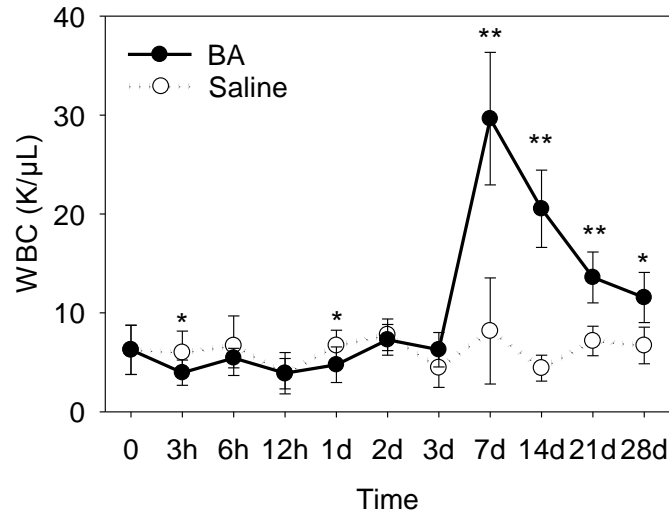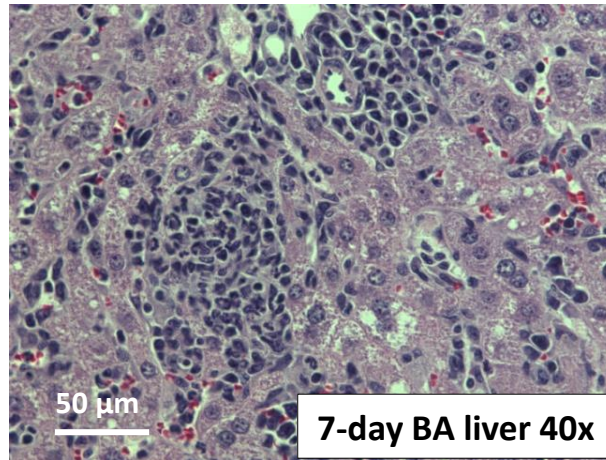
A

B

C

Figure 4-2A-C. The BA-injected mice develop both acute and chronic inflammation, with an early rise in hepcidin mRNA. Compared to saline-injected mice, the BA-injected mice develop: (A) increased SAA-1 mRNA by 3 h, peaking at 1 d, and a gradual return to normal by 21 d; (B) increased hepcidin mRNA, with a peak at 6 hours, a slight but consistent increase though 7 d, followed by significant decrease at 14 and 28 d; (C) increased serum hepcidin protein at 6 h. Panels A and B: Means ± SE, with each BA-treated group referenced to contemporaneous saline-treated group, C and D: Means ± SD, *p<0.05, **p<0.001; p by t-test or Mann-Whitney rank sum test.
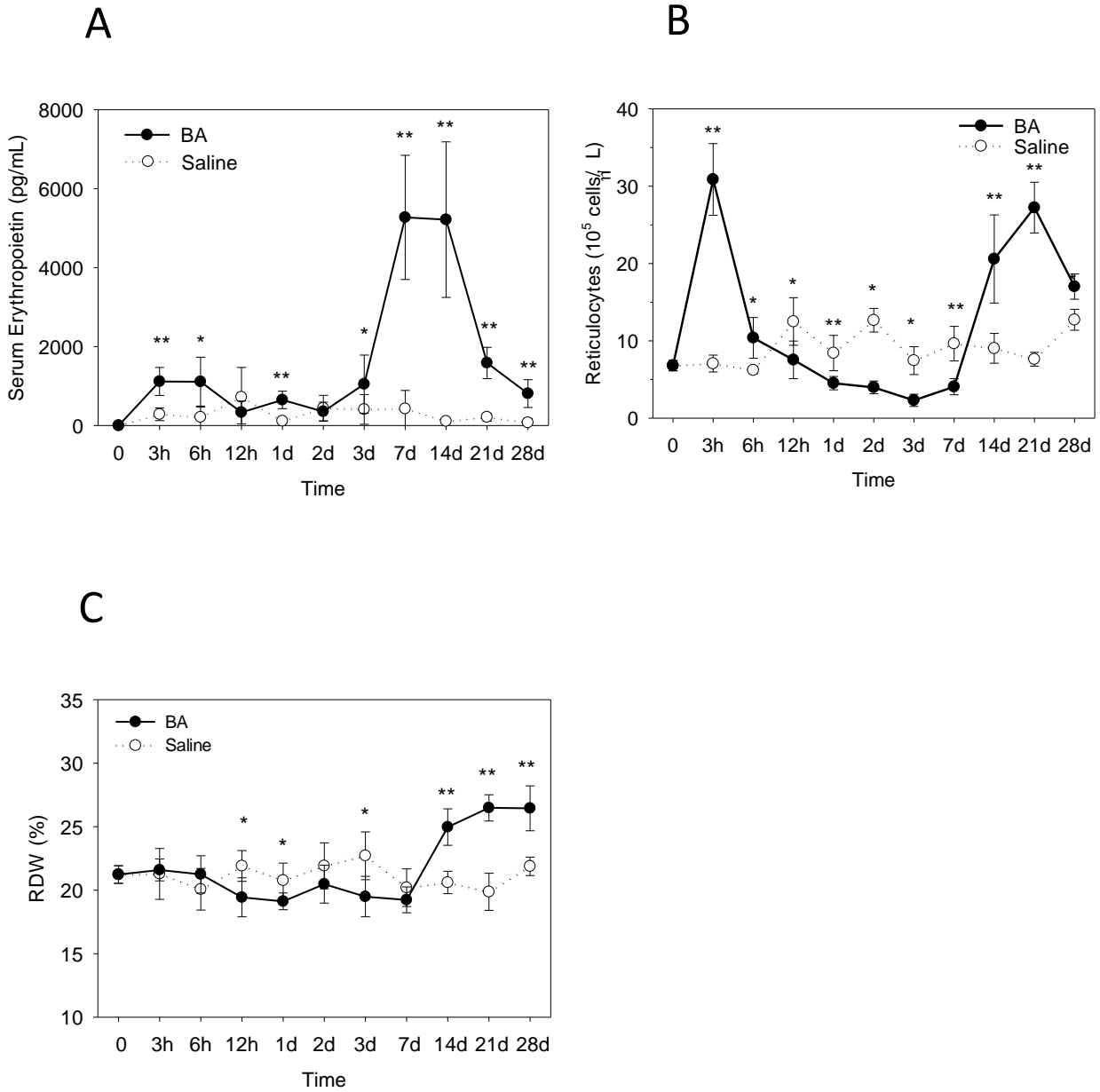
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E

Figure 4-2D,E. The BA-injected mice develop both acute and chronic inflammation, with an early rise in hepcidin mRNA. Compared to saline-injected mice, the BA-injected mice develop: (D) increased WBC by 7 d, with partial recovery by 28 d; (E) hepatic perivascular infiltration of inflammatory cells by 7 d. Means ± SD, *p<0.05, **p<0.001; p by t-test or Mann-Whitney rank sum test.

Figure 4-3. Suppression and recovery of erythropoiesis in BA-treated mice. (A) Serum erythropoietin concentrations in BA-treated mice increase to a peak by 7 d, with a decline approaching normal values by 28 d. (B) After the initial release of reticulocytes at 3 h, reticulocytes decrease significantly below those of saline-treated mice from 12 h to 7 d, followed by reticulocytosis on days 14-28. (C) Consistent with decreased reticulocytes in BA-treated mice, the red cell distribution width is suppressed at 12 h-3 d but increases by 14 d. Means ± SD are shown, *p<0.05, **p<0.001; p by t-test or Mann-Whitney rank sum test.

A
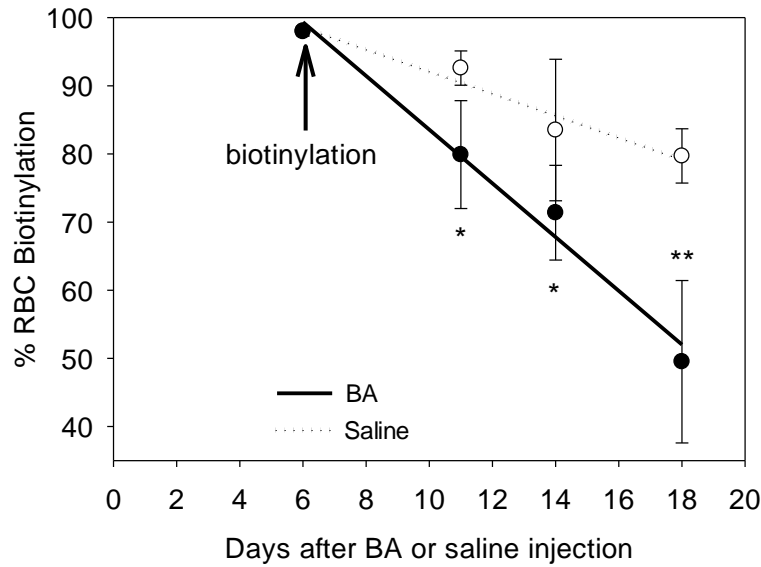
B
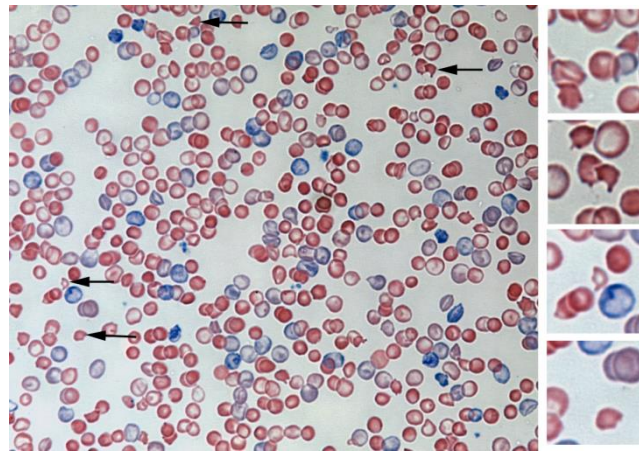
Figure 4-4. BA-treated mice have shortened RBC lifespan and evidence of hemolysis. (A) The RBC biotinylation assay indicates a 2.7-fold increase in RBC destruction in BA-treated mice (-4%/day vs -1.7%/day). Means ± SD, *p<0.05, **p<0.001; p by t-test or Mann-Whitney rank sum test. Overall difference between the two sets of measurements is significant at p<0.001 by 2-way ANOVA (Holm-Sidak method) (B) Peripheral blood smears of BA-injected mice 2 wks after show schistocytes and erythrocyte membrane irregularities, consistent with mild microangiopathic hemolysis. Arrows point to damaged erythrocytes, shown magnified in the right margin.
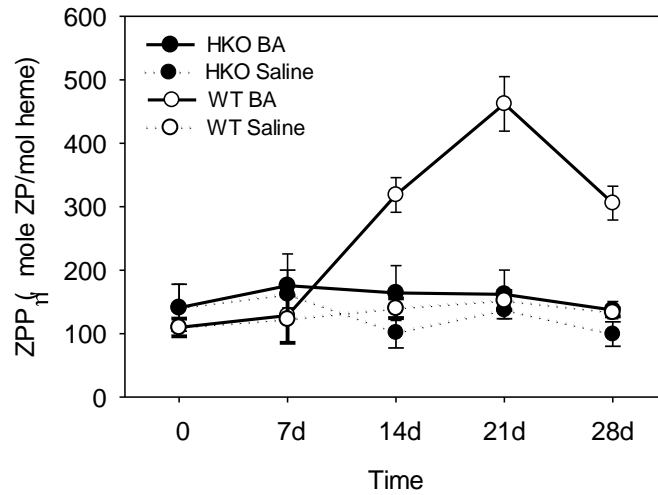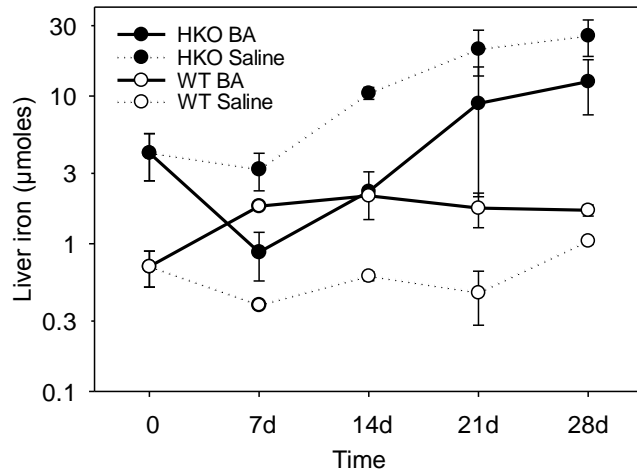
A
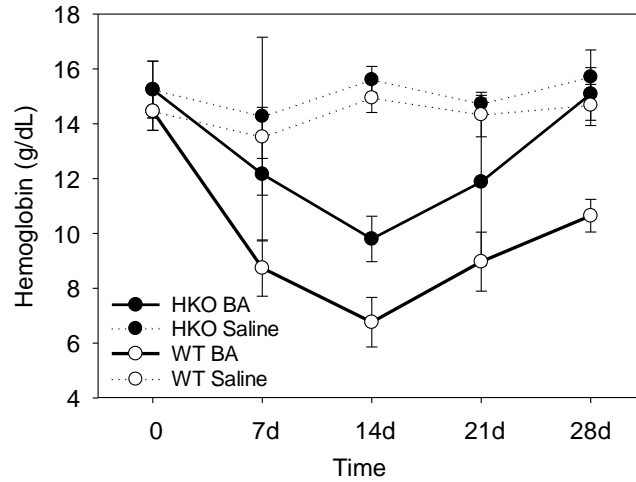
B
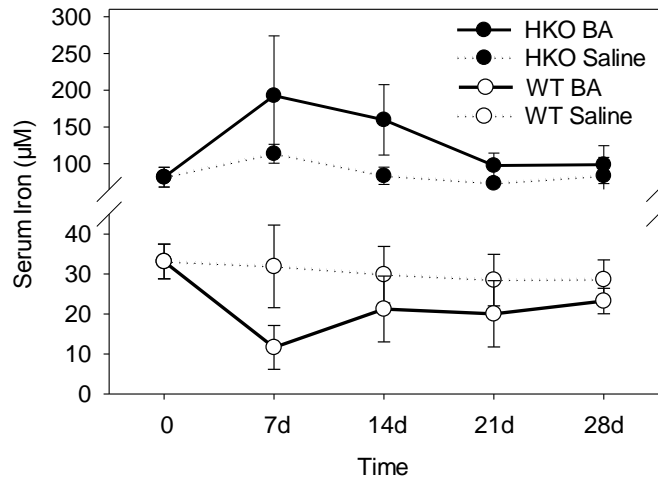
Figure 4-5A,B. Hepcidin ablation attenuates BA-induced anemia and iron restriction. (A) BA-treated
Hamp-KO mice (HKO) develop milder anemia than their WT counterparts, and completely recover by 28 d.
Hamp-KO BA vs WT BA, day 7 or 21, p<0.05; *Hamp*-KO BA vs WT BA, day 14 or 28, p<0.001, n=3-8 per
time point. (B) Unlike their WT counterparts, BA-injected *Hamp*-KO mice increase their serum iron levels.
Hamp-KO BA vs *Hamp*-KO Saline, day 14, p<0.05; WT BA vs WT Saline, days 7, 14, 21 and 28, p<0.05.
Means ± SD are shown, p by t-test or Mann-Whitney rank sum test. WT data shown in A and B are identical to
those from Figure 4-1A and B.

C

600

ZPP (µ mole ZP/mol heme)

500

400

300

200

100

0

— ● — HKO BA
···●··· HKO Saline
— ○ — WT BA
···○··· WT Saline

0   7d   14d   21d   28d

Time

D

30

Liver iron (µmoles)

10

3

1

0.3

0.1

— ● — HKO BA
···●··· HKO Saline
— ○ — WT BA
···○··· WT Saline

0   7d   14d   21d   28d

Time

Figure 4-5C,D. Hepcidin ablation attenuates BA-induced anemia and iron restriction. (C) BA-treated WT mice have dramatically increased RBC zinc protoporphyrin, indicating iron-restricted erythropoiesis, as compared to the very modest increases of ZPP in *Hamp*-KO mice. (D) BA-treated *Hamp*-KO mice mobilize iron from liver iron stores, while their WT counterparts mildly increase their stores in the face of anemia. *Hamp*-KO BA vs *Hamp*-KO Saline, day 14, $p<0.001$; day 28, $p<0.05$. WT BA vs WT Saline, days 7 or 14, $p<0.001$; day 21, $p<0.05$. Means ± SD are shown, p by t-test or Mann-Whitney rank sum test. WT data shown in C is identical to that from Figure 4-1D.

Supplemental Table 4-1: PCR Primers

| Gene target	Forward Primer	Reverse Primer
Murine HAMP1	5'-CCTATCTCCATCAACAGATG-3'	5'-AACAGATACCACACTGGGAA-3'
Murine β-Actin	5'-ACCCACACTGTGCCCATCTA-3'	5'-CACGCTCGGTCAGGATCTTC-3'
Murine SAA-1	5'-TGACCAGGAAGCCAACAG-3'	5'-GTAGGAAGACCAGACC-3'
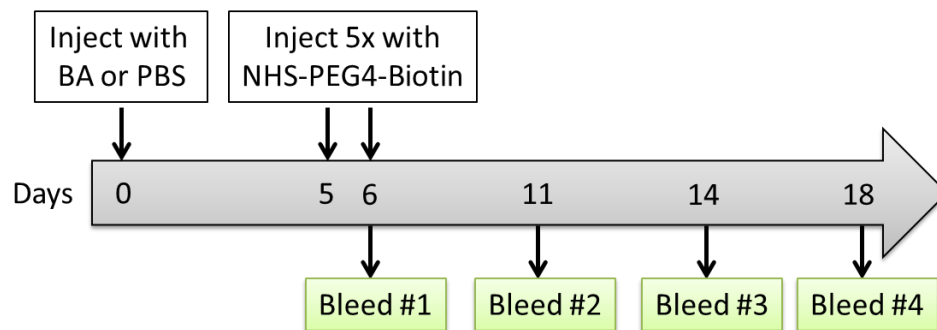
Supplemental Figure 4-1: Erythrocyte biotinylation timeline

Supplemental Figure 4-2: BA treatment causes perivascular granulomatoid inflammation with thrombosis
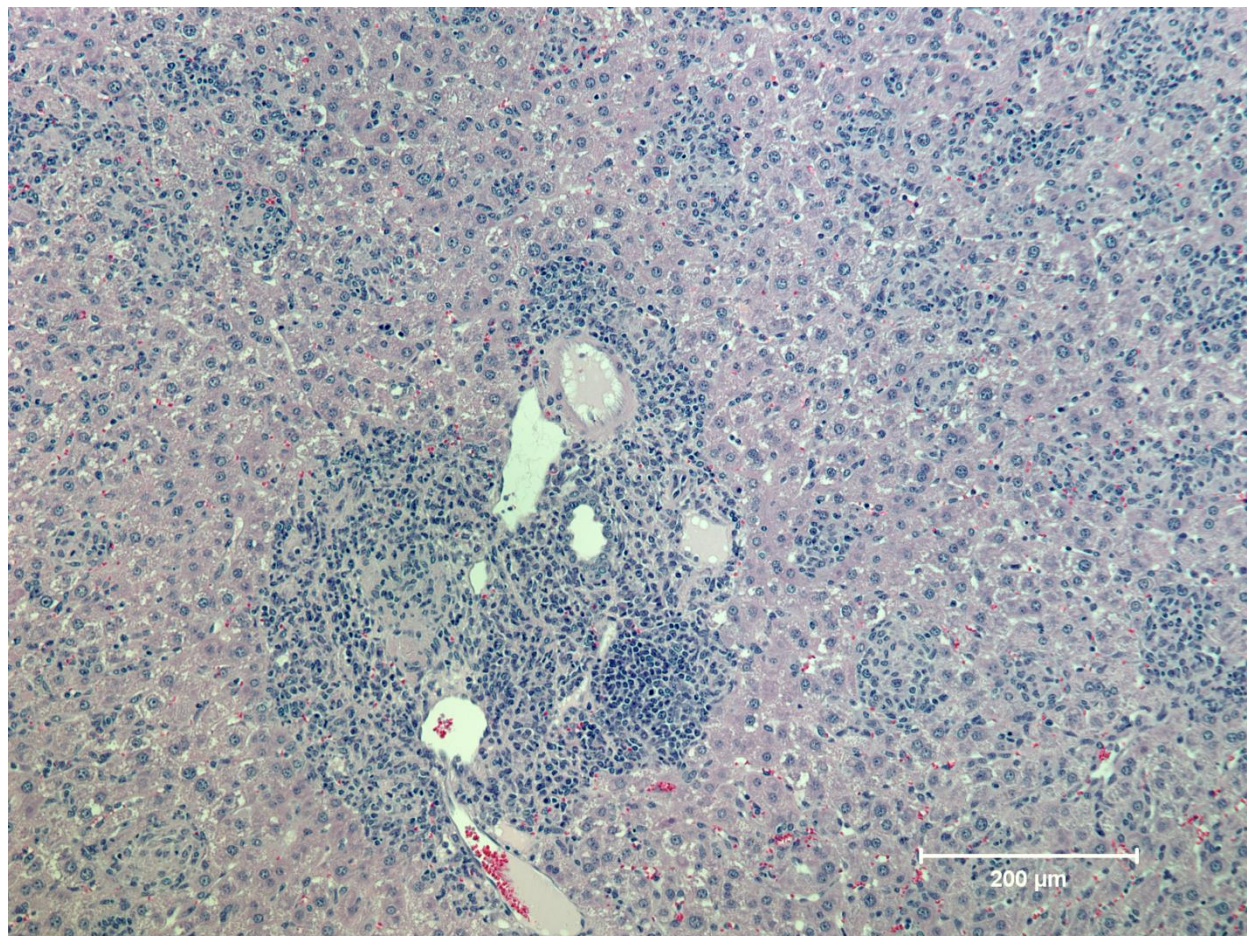
Panel A: An H&E stained liver section from BA-treated mouse at 2 wks shows granulomatoid accumulations of inflammatory cells predominantly in the periportal area.
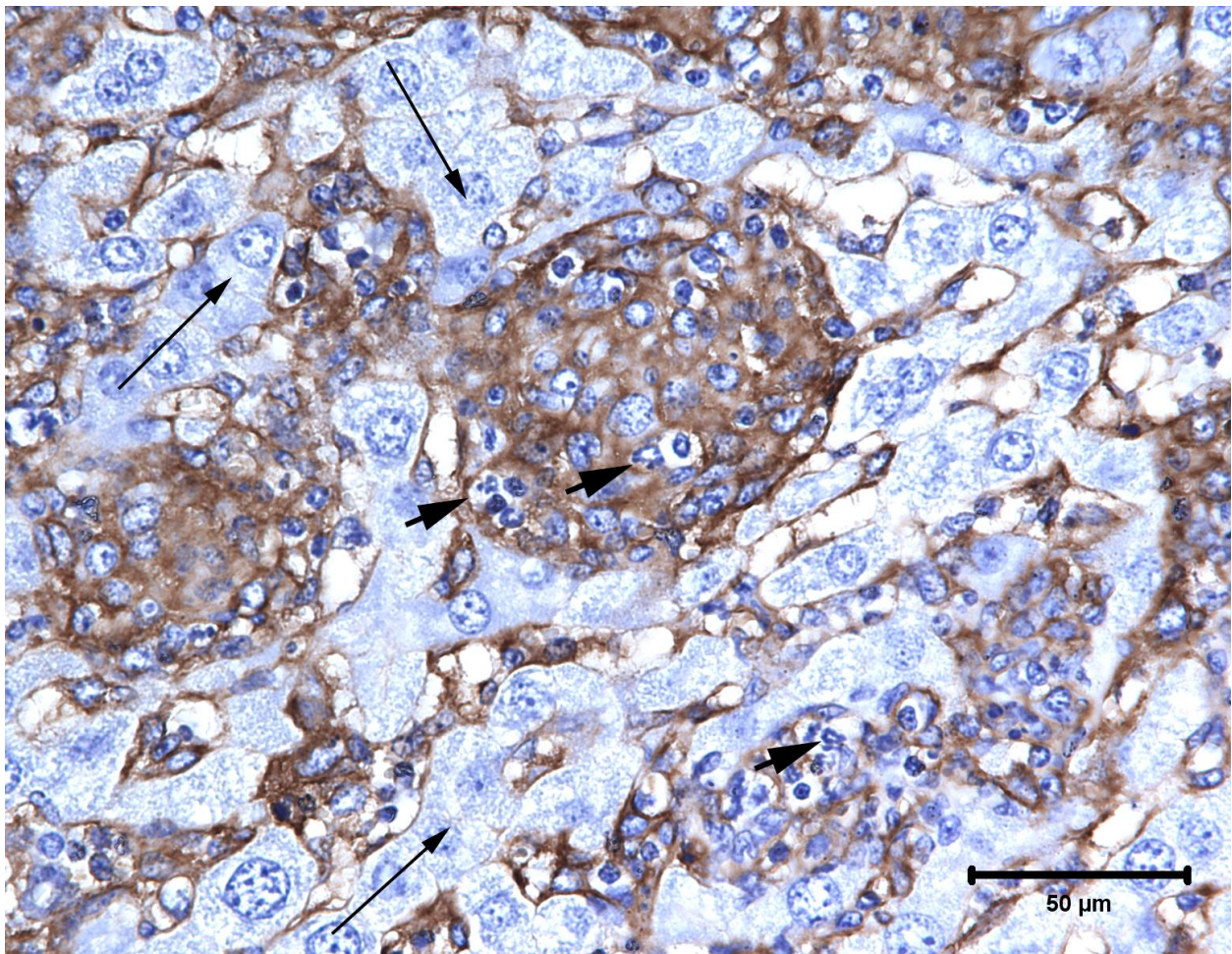
Panel B: Liver section from BA-treated mouse at 2 wks stained with a monoclonal antibody against the F4/80 macrophage marker (brown) shows granulomatoid accumulations of macrophages (brown staining predominantly on cell membranes) and granulocytes (short arrows, unstained white with multilobed blue nuclei) surrounded by cords of hepatocytes (long arrows, hematoxylin blue staining with large nuclei and reticulated cytoplasm).
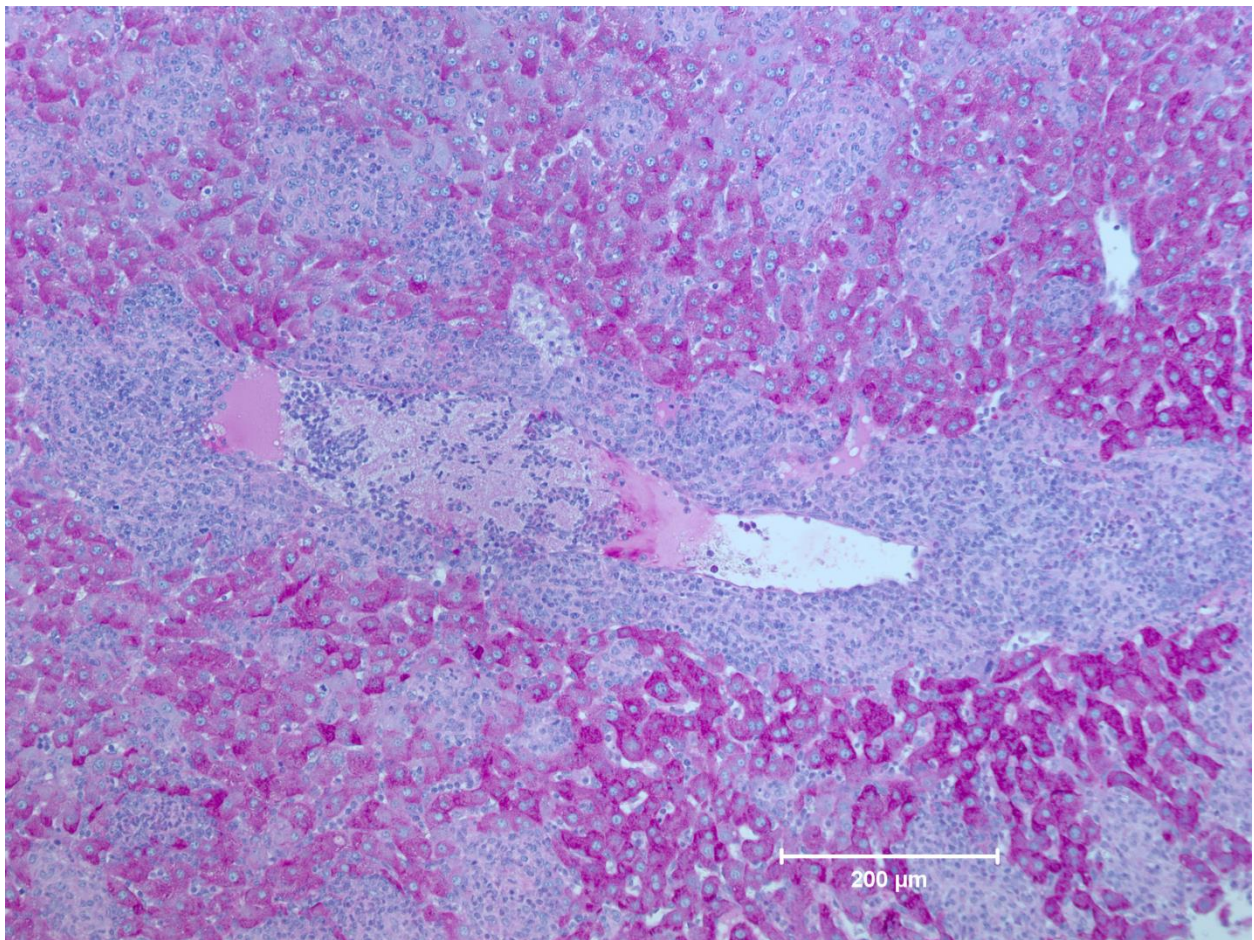
Panel C: A PAS-stained liver section from BA-treated mouse at 2 wks shows perivascular inflammation and thrombosis.
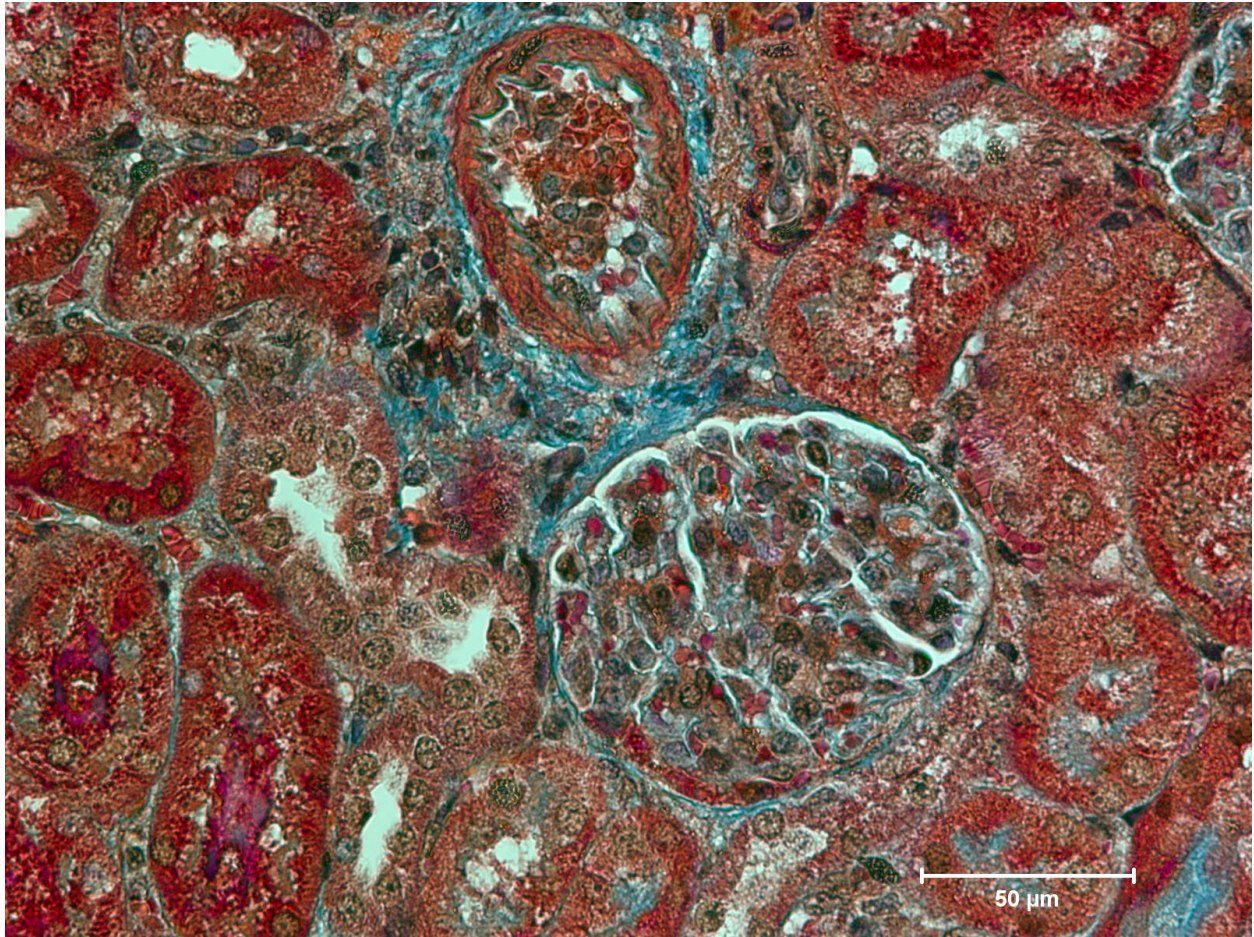
Panel D: A Masson-trichrome stained kidney section from a BA-treated mouse at 2 wks shows periarteriolar inflammatory cells with arteriolar thrombosis and a hypoperfused glomerulus.
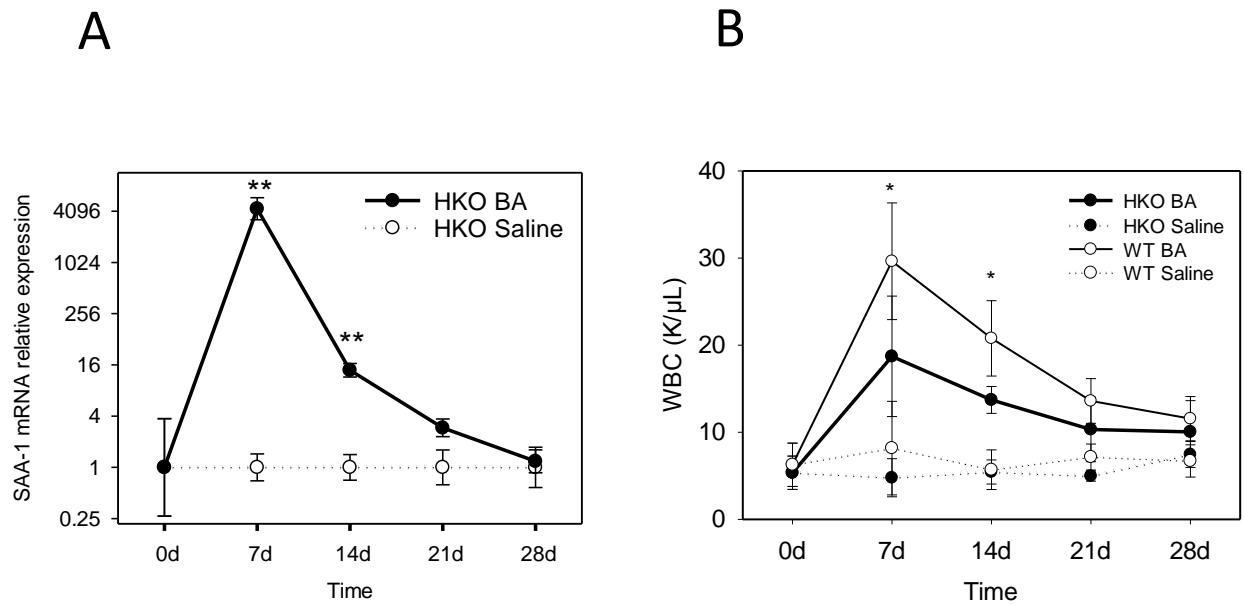
Supplemental Figure 4-3: Inflammatory markers in HKO mice. A: Hepatic SAA-1 mRNA concentrations in BA-treated HKO mice relative to those of contemporaneous saline-treated HKO mice. HKO mice have increased SAA-1 mRNA, with a peak at 7 d and a gradual return to normal by day 21. Means ± SE are shown, p by t-test or Mann-Whitney rank sum test. **p<0.001.

B: WBC (means ± SD) of HKO and WT mice, *p<0.05 by t-test, comparing BA-treated HKO to BA-treated WT. The data shown for WT mice are a subset of those in Figure 4-2D.
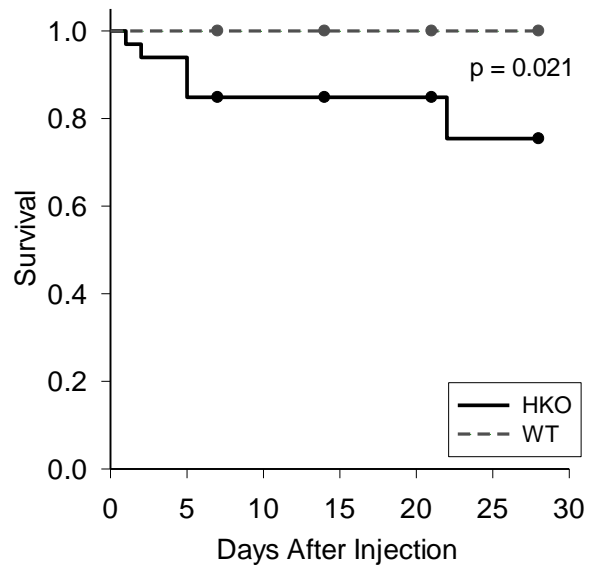
Supplemental Figure 4-4: HKO mice have significantly decreased survival compared to their WT counterparts. Kaplan-Meier survival analysis with p by log-rank test.

Experimental validation of the Amgen sandwich ELISA for mouse hepcidin-1

We tested whether the hepcidin-1 ELISA appropriately detected the predicted effect of dietary iron on serum hepcidin-1 concentrations. Serum hepcidin-1 concentrations were also correlated with hepatic hepcidin-1 mRNA, the current gold standard for studies of hepcidin responses in mice.

Materials and Methods

Animals

Four week old C57BL/6 mice (8 males and 8 females per diet group) were placed on 3 different iron-containing diets for one month. One set of mice was put on a low iron diet containing 4ppm iron (Harland Laboratories, Indianapolis, IN, TD80396) for 4 weeks, a second set was kept on the regular mouse chow which contained approximately 300 ppm iron. The third set of mice was maintained on the regular diet for one week then changed to a high iron (carbonyl) diet containing 10,000 ppm iron (Harland Labs, TD08043) for the remaining three weeks.

Sample collection

Approximately 100 µl blood was collected in microtubes containing 3 µl of K_3-EDTA and mixed immediately. Complete blood count was performed by a hematology analyzer (Hemavet HV950FS, Drew Scientific, Dallas, Texas).

Additional blood was allowed to clot in a serum separator. The serum was collected after centrifugation at 5000 RPM for 5 minutes, and stored at -20C until used for the ELISA. Liver tissue was collected and analyzed for HAMP mRNA using qRT-PCR.

ELISA

Mouse hepcidin-1 monoclonal antibodies, Ab2B10 (capture) and AB2H4-HRP (detection), as well as synthetic mouse hepcidin-25, were a generous gift from Amgen (Thousand Oaks, CA). A protocol[36] that was designed by Dr. Keegan Cooke and Dr. Barbara Sasu at Amgen was used as the starting point for further development and validation of this assay.

High binding 96-well EIA plates (Corning Costar, Tewksbury MA, #3590) were coated overnight at room temperature with 50 μl/well of 3.6 μg/ml Ab2B10 in 0.2 M carbonate-bicarbonate buffer pH 9.4 (Pierce - Thermo Scientific, Rockford, IL). Plates were washed two times with wash buffer (PBS, 0.5% Tween-20) and then blocked for 45 minutes with 200 μl/well blocking buffer (PBS, 1% BSA, 1% normal goat serum, 0.5% Tween-20). Samples and standards were then placed in the wells in duplicate. A standard curve was generated by diluting the stock mouse hepcidin peptide (50 ng/ul) to a final concentration of 4 ng/ml for the highest standard followed by two-fold dilutions in blocking buffer, thereby generating an 8-point standard curve. Serum samples from mice on a high iron diet were diluted approximately 1:5000, whereas those on a low iron diet, as well as the hepcidin knock-out control serum were diluted 1:100. After a one hour incubation period at room temperature (with mixing), the wells were washed four times with wash buffer then incubated for an additional hour with 50 μl/well of 130 ng/ml Ab2H4-HRP, washed 4 times then developed with 100 μl/well Ultra-TMB substrate (Thermo Scientific) for 30 minutes in the dark at room temperature. The reaction was stopped by adding 50 μl 2 M sulfuric acid and the absorbance was measured at 450 nm using a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

qRT-PCR:

Total RNA was extracted from mouse liver tissue using the Trizol method (Invitrogen, Grand Island, NY) according to manufacturer's instructions. cDNA was made using the BioRad iScript cDNA kit (Hercules, CA) using approximately 250 ng total RNA using the following conditions; 25C for 5 min, 42C for 30 min, 85C for 5 min. BioRad IQ SyberGreen supermix was used for qRT-PCR reactions with a two-step program set at 95C for 10 sec and 60C for 30 sec for 35 cycles. The following primer sets were used:

Mouse Hepcidin: FWD: 5'-AAGCAGGGCAGACATTGCGAT-3'

                         REV: 5'-CAGGATGTGGCTCTAGGCTAT -3'

Mouse Actin: FWD: 5'-ACCCACACTGTGCCCATCTA-3'

                         REV: 5'-CACGCTCGGTCAGGATCTTC-3'

Results

There was no significant difference in the blood hemoglobin concentration among the three groups (12.9±3.0, 13.3±3.1, 12.9±3.3 g/dl for low, standard and high iron diets respectively). As expected for iron-restricted erythropoiesis, the MCV of the low iron group was lower (35.7±1.3, mean ± SD, p=10^{-10}, t-test compared to standard diet) than that of standard and high iron groups (40.6±1.6 and 41.6+1.6 respectively).

Hepcidin concentrations in the serum of mice on the low iron diet were mostly below the lower limit of detection (3 ng/ml) in 1:100 dilution of serum. This minimum serum dilution was determined by comparing hepcidin-1 peptide standard curves generated in various dilutions of hepcidin knock-out (HKO) mouse serum to those generated in blocking buffer (BB) alone (Supplemental Figure 4-5), showing nonspecific interference at serum dilutions below 1:50.

Increasing concentrations of hepcidin were seen with increasing amounts of iron in the diet (Supplemental Figure 4-6). Dietary iron ranged over a 10,000 fold range and hepcidin also increased over a similar range (3-7000 ng/ml). On the 300 ppm (standard mouse chow) diet, females had roughly two-times higher serum hepcidin concentrations than males, median (interquartile range) 1825 (1705, 1988) ng/ml vs. 938 (700, 1275) ng/ml, $p<0.001$ by Mann-Whitney, whereas females and males had approximately the same concentrations of hepcidin on the high iron diet, 4863 (4541, 5224) ng/ml and 5055 (4406, 6200) ng/ml. Replicate analysis for this assay showed a coefficient of variation of 9% in the same plate and 19% for replicates in separate plates over the range of the assay (3-7000 ng/ml).

As shown in Supplemental Figure 4-7, liver hepcidin mRNA (normalized to actin mRNA) was very low in the low iron diet mice dCt median (interquartile range) females -7.5 (-7.8, -4.7) and males -6.1 (-7.5, -4.5), $p=0.65$ by Mann-Whitney test, whereas on the 300 ppm diet, females had 2-fold more hepcidin mRNA compared to male mice dCt 3.3 (2.7, 4.1) and 2.3 (1.8, 2.8) respectively, $p=0.02$ by Mann-Whitney. Like hepcidin peptide concentrations, the hepcidin mRNA concentrations were very similar between the female and male mice on the high iron diet 4.2 (3.9, 4.4) and 4.3 (3.9, 5.0), $p= 0.72$ by Mann-Whitney test.

There was a very strong correlation ($r^2 = 0.91$, Pearson) between log serum hepcidin concentration and liver mRNA levels (Supplemental Figure 4-8), supporting the validity of the new serum hepcidin assay, and reinforcing the validity of hepatic hepcidin mRNA concentrations as a proxy for the less accessible serum hepcidin peptide measurements.
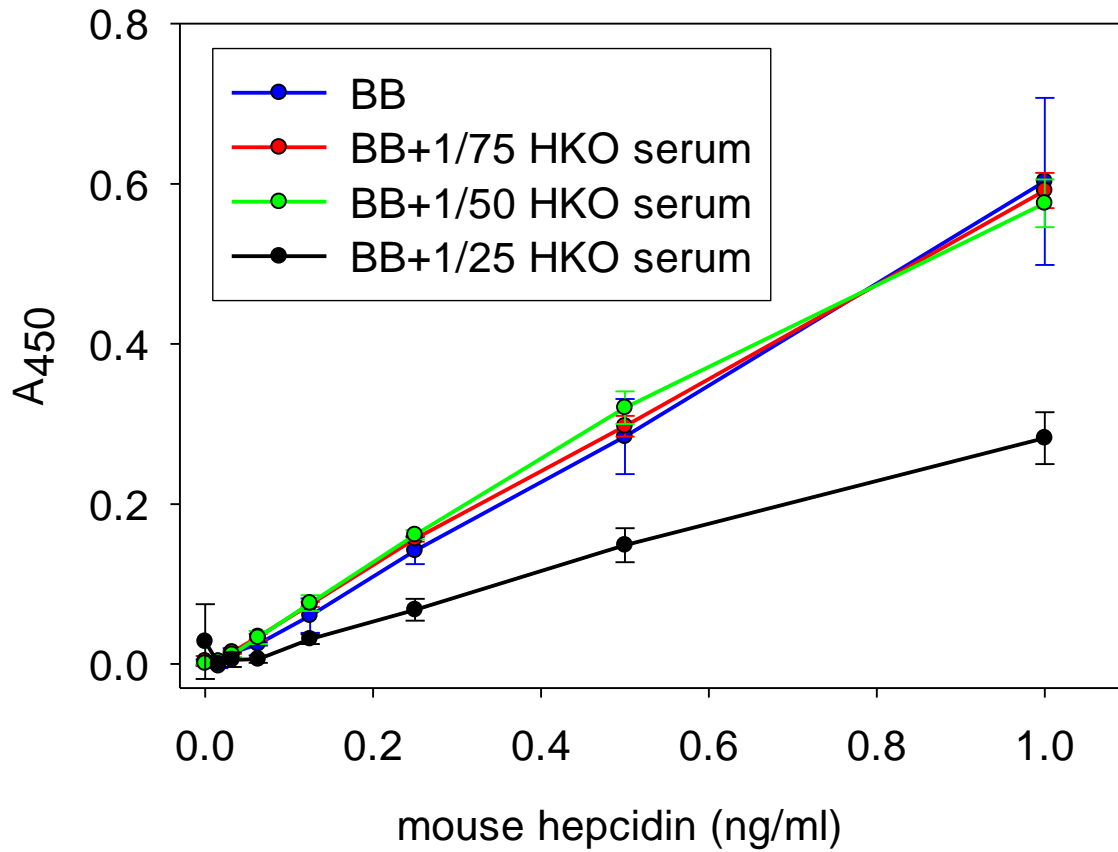
Supplemental Figure 4-5. Mouse hepcidin standard curves in dilutions of hepcidin-knock-out mouse serum (HKO). Standard curves of mouse hepcidin peptide in different concentrations of hepcidin-knockout (HKO) mouse serum diluted in blocking buffer (BB) were tested to determine the lowest concentration of serum that does not cause interference (1:50). Standard curves are shown with means ± SD of replicate measurements.
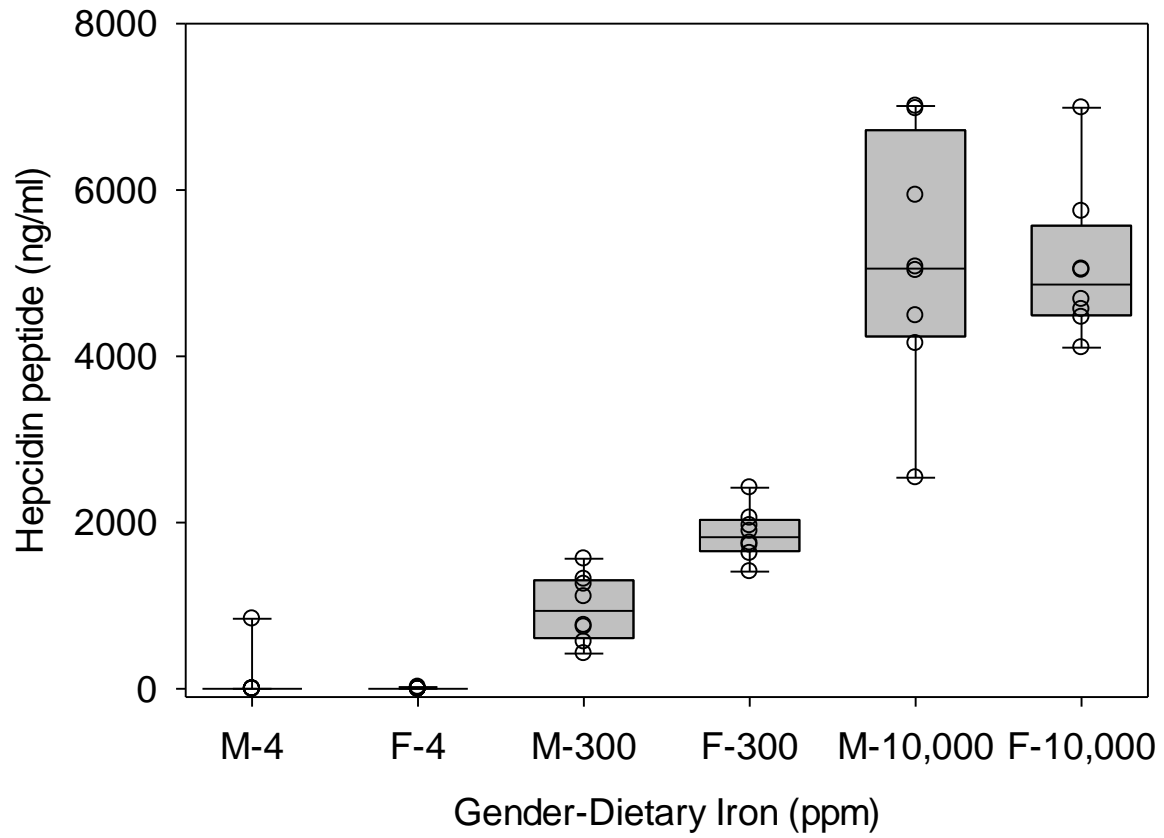
Supplemental Figure 4-6. Hepcidin peptide concentrations (ng/ml) in sera of male (M) and female (F) mice fed diets with different iron contents (4 ppm, 300 ppm or 10,000 ppm) for one month. Median and interquartile range are shown in the box plot and individual data points are shown as hollow circles.
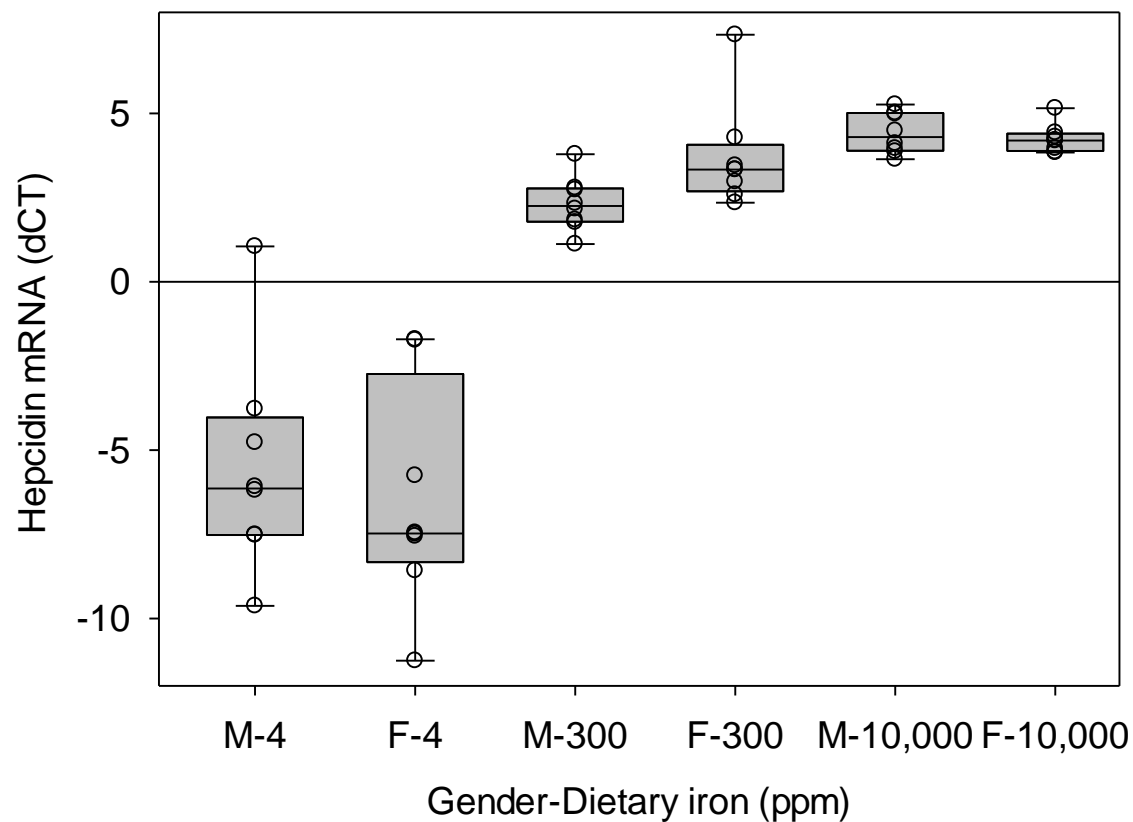
Supplemental Figure 4-7. Hepcidin Liver mRNA (dCT) in Male (M) and Female (F) mice on different iron containing diets (4ppm, 300ppm or 10,000ppm) for one month. Hepcidin and actin cDNA was amplified using qRT-PCR and Hepcidin mRNA normalized to actin. Median and interquartile range are shown in the box plot and individual data points are shown in the hollow circles.
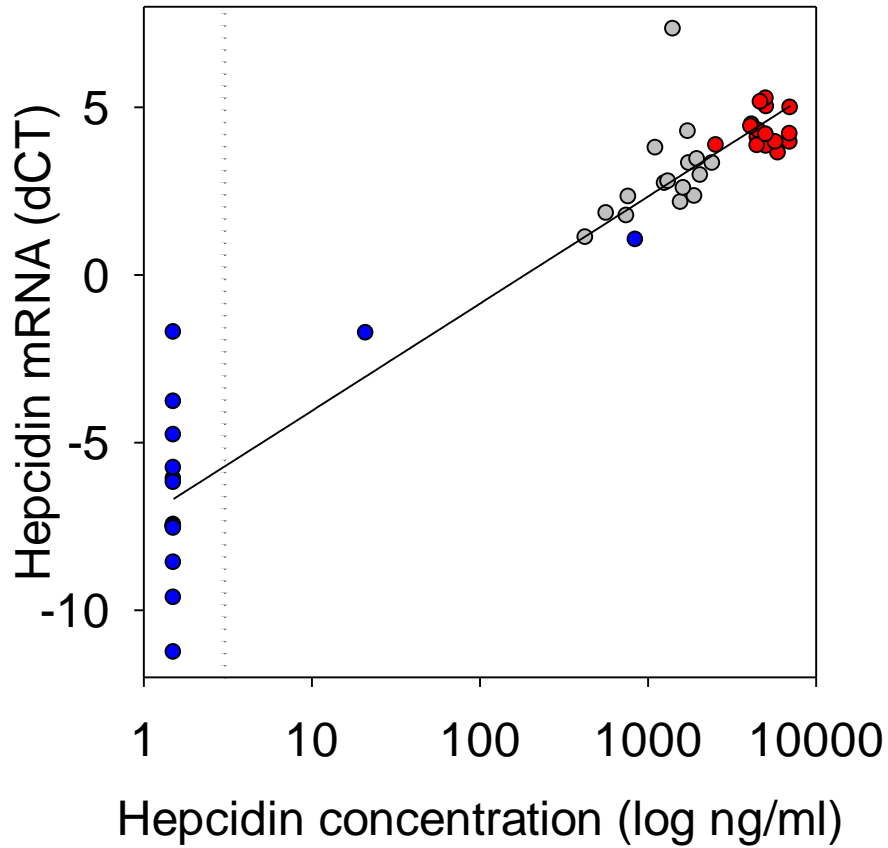
Supplemental Figure 4-8. Correlation of liver hepcidin-1 mRNA concentrations with serum hepcidin-1 peptide concentrations. Value for individual mice on different iron containing diets are indicated by the colors: blue = 4 ppm , gray = 300 ppm and red =10,000 ppm. Serum hepcidin concentrations not detectable by the EIA were assigned a concentration of 1.5 ng/ml, representing 50% of the lower limit of detection (dotted vertical line).

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Chapter 5

CONCLUDING REMARKS

Anemia of inflammation (AI) is a widely prevalent condition in patients that are suffering from other primary pathologies. Although anemia has been shown to be a negative prognostic indicator for morbidity and mortality in a variety of inflammatory conditions, the traditional therapy has been to treat the underlying disease process. When this is not achievable, the mainstays of therapy have been iron supplementation, red blood cell transfusions, and erythropoiesis-stimulating agents. Unfortunately, concerns about the safety and efficacy of these treatments have limited their use in the clinical realm. Red blood cell transfusions carry the risks of immunosuppressive effects, transmission of infections, and transfusion reactions [1], and has been shown to be an independent risk factor for increased mortality in ICU patients [2]. Erythropoiesis-stimulating agents (ESAs) has been associated with higher mortality rates in patients with AC [3], and with deep venous thromboses in ICU patients with negligible decrease in transfusion requirements [4]. Because of the recent discovery of the iron-regulating peptide hormone hepcidin, there has been much interest in investigating hepcidin as a potential therapeutic target in AI.

This dissertation has described the use of mouse models to characterize the inflammatory anemias that occur secondary to primary inflammatory conditions, and to investigate the role of hepcidin. Anemia of aging (AA), anemia of cancer (AC), and ICU anemia are three phenomena that share the basic criteria of AI, but differ significantly in their chronicity and severity. We explored the pathogenesis of AA (chronic and mild), AC (varying acuity and severity), and sepsis anemia (acute and severe) by utilizing mouse models of disease. Along with a detailed characterization, each set of experiments employed the use of hepcidin KO mice to further investigate the role of hepcidin.

In Chapter 2, we have used wild-type (WT), hepcidin KO, and IL-6 KO mice to investigate the roles of IL-6 and hepcidin in AA. WT mice developed a relative anemia with aging. Examination of iron parameters showed that the aged WT mice had hypoferremia with increased tissue iron stores. Given the similarity to the AI phenotype, we also examined inflammatory and hepcidin parameters. Hepcidin measurements revealed a non-significant trend towards increased expression in aged WT mice. However, this level of hepcidin would be considered inappropriately high in the face of anemia and erythropoiesis, which suppress hepcidin production. We hypothesized that IL-6 activity was contributing to the relatively increased hepcidin expression. Of the inflammatory cytokines that were measured, IL-6 and IFN-γ levels were significantly increased in the aging WT mice. When the experiment was performed in aged IL-6 KO mice, we found that this group of mice also had a drop in their hemoglobin as they aged. However, in the group of retired breeder IL-6 KO mice, this drop in hemoglobin was significantly smaller than the drop in WT mice. The aged hepcidin KO mice also had a drop in their hemoglobin as they aged, but this drop was also significantly smaller in magnitude than in WT mice. Thus, IL-6 and hepcidin ablation both appeared to be partially protective against the anemia of these aging mice. Examination of erythropoiesis in the aged mice showed that IL-6 KO mice had more intramedullary erythropoiesis than in WT mice, implying that IL-6 plays a role in the impaired erythropoiesis of aging. In conclusion, this mouse model of aging demonstrated the characteristic iron profile of anemia of inflammation in WT mice. The aged mice also had increased IL-6 levels, which is seen in their human counterparts [5], and relatively increased hepcidin expression. IL-6 ablation and hepcidin ablation both attenuated the drop in hemoglobin that occurred with aging, and IL-6 ablation was protective against the inefficient

erythropoiesis in aging mice. Thus, although IL-6 and hepcidin do not appear to be required for the development of anemia in this aging mouse model, they do play a significant role.

Chapter 3 was the description of four different mouse models of AC: melanoma, ovarian cancer, and two types of lung cancer. These models generated either rapidly-growing or slow-growing tumors. The melanoma model developed a rapid and severe anemia, with marked iron-restricted erythropoiesis. Evaluation of inflammatory parameters confirmed that this was an inflammatory anemia, although hepcidin mRNA levels were not elevated at the time point evaluated. Rather, hepcidin was suppressed, likely as a response to anemia. One of our rapidly-growing lung cancer models developed a mild anemia, while the second model developed a severe anemia. However, both displayed features characteristic of AI: iron-restricted erythropoiesis, intact tissue iron stores, and increased inflammation. The milder model also had an increase in hepcidin production, perhaps secondary to reduced suppressive effect of erythropoiesis as compared to the more severely anemic lung cancer model. Our slow-growing ovarian cancer model also developed a significant anemia with iron restriction. However, these mice had reduced tissue iron stores that were consistent with frank iron-deficiency anemia. When the milder lung cancer model and the ovarian cancer model were repeated in hepcidin KO mice, we found that there was no difference in the hemoglobin drop in either model at the single time point evaluated for each model. In conclusion, these cancer models demonstrated a spectrum of anemia that also occurs with malignancies in human patients. Three out of the four models developed an inflammatory anemia, while the fourth and most chronic model developed a frank iron-deficiency anemia which was likely secondary to hemorrhagic ascites and nutritional deficiencies. Only one of the four models had an increase in hepcidin levels, although we cannot rule out the possibility that the other models had inappropriately elevated hepcidin expression

given their degree of anemia and erythropoiesis. Finally, the cancer models that were repeated in hepcidin KO mice did not show any perceptible contribution of hepcidin to AC. While this does not completely eliminate the possibility of a partial role of hepcidin, there are clearly other etiologies at play.

Chapter 4 is a detailed characterization of a *Brucella abortus* mouse model of acute inflammation. These mice developed a severe anemia with the characteristic findings of AI. They had hypoferremia and evidence of iron-restricted erythropoiesis despite iron accumulation in the liver. There was also increased erythrocyte destruction as evidenced by schistocytes on peripheral blood smears, and a shortened RBC lifespan per an RBC biotinylation assay. Given the relative paucity of schistocytes, we hypothesize that the shortened lifespan was likely secondary to increased erythrophagocytosis by cytokine-activated macrophages. There was also suppression of erythropoiesis despite adequate circulating erythropoietin levels, as has been described in other inflammatory anemia studies [6]. Libregts et al. used a different inflammatory mouse model to demonstrate that chronic IFN-γ production promoted monocytic differentiation with a side effect of erythroid suppression [7]. Evolutionarily, this strategy during times of infection would maximize the production of monocytes and macrophages necessary to fight infection. Interestingly, our *Brucella*-injected hepcidin KO mice had an attenuated anemia and a milder leukocytosis, accompanied by an increase in mortality. A second etiology for the blunted erythropoiesis in our *Brucella*-treated mice would be hepcidin-mediated iron restriction. In times of iron restriction, aconitase activity increases to convert citrate to isocitrate, which leads to the inhibition of the transcription factor PU.1 and stimulates erythropoiesis. Conversely, hepcidin-mediated iron restriction would have the opposite effect of suppressing erythropoiesis [8]. Thus, we hypothesize that the blunted erythropoiesis of our mouse model is caused by both increased

IFN-γ activity and hepcidin-mediated iron restriction. When the *Brucella abortus* injection was repeated in hepcidin KO mice, the anemia was significantly milder with faster recovery. In comparison to the WT mice, there was also increased iron availability and mobilization from tissue stores. This mouse model manifests the multifactorial pathogenesis of AI, demonstrates the contribution of hepcidin, and will likely be a useful tool for future research in the field of AI.

In conclusion, we have characterized mouse models of anemia of aging, anemia of cancer, and anemia of sepsis, in order to elucidate the role of hepcidin and provide a research tool for potential future therapeutics.

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