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

 doi:10.3324/haematol.2013.087114
The Online version of this paper contains a Supplementary Appendix.
Manuscript received on February 28, 2013. Manuscript accepted on August 30, 2013.
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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 approximately12 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, keratinocyte-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

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

Parameter[$]	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[&]
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

[$]±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; [&]2-way factorial ANOVA.

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

Parameter[$]	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[&]
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

[$]± 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; [&]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).

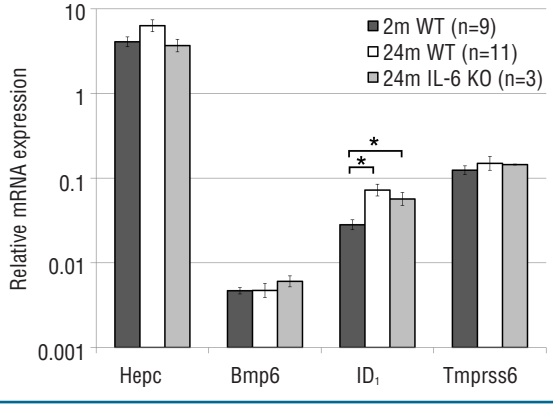

Figure 1. Relative mRNA expression of members of the Hepc regulatory pathway in the liver of young and aged WT and aged IL-6 KO mice. We assessed the fold change relative to internal housekeeping genes for each target gene and each group of mice. This relative mRNA expression of 2 month (m) WT (dark 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*).

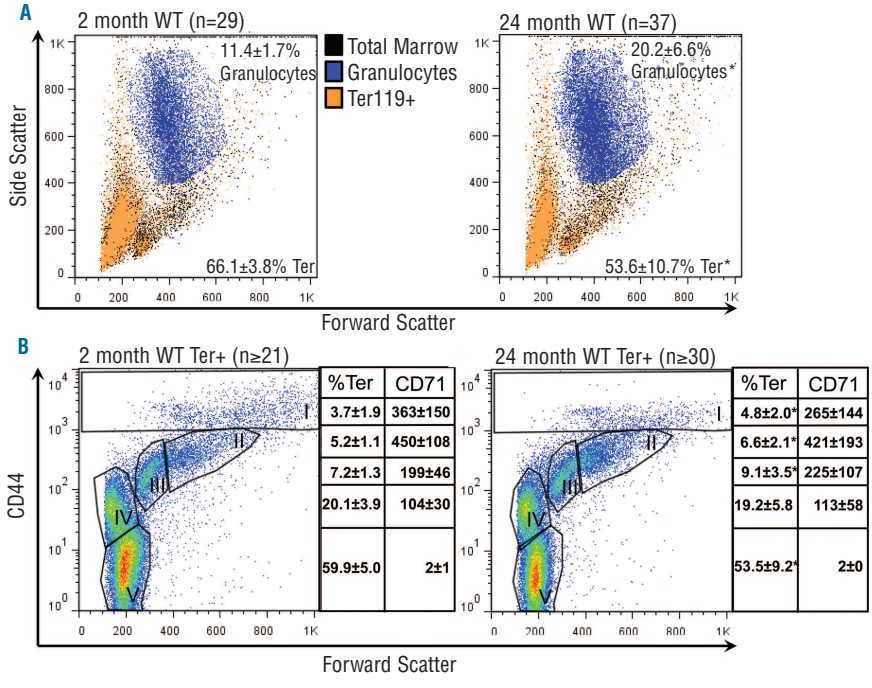

Figure 2. Erythroid maturation is impaired in aged WT mice. (A) We assessed the percentage of erythroid progenitors [Ter119 positive (Ter), back gate appears in orange] in the bone marrow of 2m (left panel) or 24m (right panel) WT mice. We found granulocyte progenitors (events with high forward scatter 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 (CD44^{high}: I, II, and III) progenitors in the aged mice (right panel) and a significant decrease in the percentage of latest stage erythrocytes (CD44^{low}). 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 concentration 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)[$]	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; $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*

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.

Acknowledgments

The authors would like to thank Victoria Gabayan and Ian Prince for their assistance in the development of the aged mouse colonies.

Funding

This work was supported by RO1 DK082722, a research career development award from P30 AG021334, the American Society of Hematology Scholar's Award, and the Nathan W. and Margaret T. Shock Aging Research Foundation Award to CNR and by R01 HL091531 to TG; BJM was supported by T32 AG000120; ODP was supported by a grant of the University Hospital of Basel, Switzerland and the Freiwillige Akademische Gesellschaft Basel, Switzerland; LKF was supported by the Minority Medical Student Award Program of the American Society of Hematology; SR was supported by K08 DK074284 and the American Society of Hematology-Harold Amos Medical Faculty Development Program. Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institute of Health under Award Number P30AR053503.

This publication was also made possible by Grant Number UL1 RR 025005 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NIH or its institutes.

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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