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

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Supplementary Design and Methods

Animal Care for C57BL/6 and IL-6 KO mice (Johns Hopkins University colony): Helicobacter hepaticus negative, female mice were housed in ventilated racks (Allentown Caging Equipment) with a 14 hour light cycle at the Johns Hopkins University (JHU) specified pathogen-free barrier facility. Retired female (RB) C57BL/6 (WT) or B6.129S2-Il6tm1Kopf/J [1], (stock 2650, IL-6 KO) breeders appear in Table 1 and Table 4 of the main manuscript and Supplementary Tables S1 and S5. No data were available concerning the number of litters born to the retired female breeders. Virgin female (VF) WT or IL-6 KO mice appear in Table 2 and Table 4 of the main manuscript and Supplementary Tables S1 and S5. Food and water were provided ad libitum until 16 hours before sacrifice, at which point the mice were transferred to a clean cage and fasted overnight with only water available ad libitum. Mice were maintained on the 2018SX Teklad Global 18% Protein Extruded Rodent Diet (Harlan Teklad, Madison, WI) which contains 225 parts per million (ppm) iron. Mice with visible or palpable tumors, ulcerative dermatitis, or cachexia were euthanized before 24 months of age and excluded from the analysis.

At necropsy, one VF WT mouse appearing in Tables 2 and 4 of the main manuscript and Tables S1 and S5 was found to have hydronephrosis and second mouse from the same group was found with an abdominal tumor. Exclusion of these animals did not affect the magnitude or the significance in erythrocyte parameters with age. No IL-6 KO mice appearing in these tables were found with tumors.

For mice appearing in Figure 1 and Table 4 of the main manuscript, RB and VF mice contribute to the values for 24 month old mice, except there were no IL-6 KO VF represented in the qPCR, liver or spleen tissue iron analyses, reticulocyte analysis or Ter+ splenocytes (please see Supplementary Table S5). IL-6 KO VF were represented in the spleen weights and Ter+ Marrow in Table 4.

Animal Care for C57BL/6, IL-6 KO, and Hepc KO mice (University of California Los Angeles colony): WT mice appearing in Table 3 of the main manuscript were born to Jackson Laboratory-derived breeders in the UCLA facility. Hepc KO mice appearing in Table 3 of the main manuscript were born to mice with targeted deletion of the Hepcidin 1 gene [2] which had been backcrossed 10 generations onto the C57BL/6 background. IL-6 KO mice appearing in Supplementary Table S2 were born to Jackson Laboratory-derived breeders in the UCLA facility. All strains housed in the UCLA facility were maintained on either NIH 31 rodent diet (iron content 336 mg/kg; Harlan Teklad, Indianapolis, Indiana, USA) or Prolab RMH 2000 breeder diet (iron content 440 mg/kg; PMI Nutrition International Inc., Brentwood, Missouri, USA).

At necropsy, 3 of 10 WT mice appearing in Table 3 of the main manuscript and Supplementary Table S2 were found with tumors at necropsy. Exclusion of these animals did not affect the magnitude or significance of the decline in erythrocyte parameters with age. No IL-6 KO or Hepc KO mice appearing in these tables were found with tumors. Mice that died before 17 months of age were excluded from the analysis.

Blood collection: For longitudinal time points, approximately 100 microliters (mcl) of whole blood was collected for complete blood count from the facial vein using a lancet without anesthesia or from the retro-orbit with isofluorane anesthesia. As a terminal procedure, whole blood was collected from the retro-orbital space of mice anesthetized with intraperitoneal (IP) injection of Avertin (125-240 mg/kg). Only 50 mcl of blood was collected from mice that were analyzed for liver gene expression, to reduce the possibility of transcriptional changes resulting from hypoxia. Due to limitations in blood sample volume, it was not possible to perform every assay on serum samples from every mouse.

Enzyme-linked immunosorbent assays: Whole blood samples were allowed to clot in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA) approximately 30 minutes. Then serum was collected according to the manufacturer's specifications and immediately frozen at -80ºC. The 7-plex ELISA used to measure the cytokine IL-6 reported values above background for the IL-6 KO mice. We conclude that the ELISA probe may bind to incomplete IL-6 proteins due to the insertion of a stop codon in the

middle of the IL-6 gene. PCR-based genotyping analysis confirmed the IL-6 KO genotype of aged RB mice (not shown).

Flow cytometry: Erythroid maturation was determined in bone marrow and spleen samples as described [3] with antibodies to Ter119, CD45, CD71, and/or CD44 (BD Pharmingen, Franklin Lakes, NJ). The cell staining was quantified with the FACS Calibur (Beckton Dickenson). 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 (a marker of iron deficiency) in the individual progenitor populations.

Quantitative real-time PCR: Total RNA was isolated from snap frozen mouse livers by the Lowe Family Genomics Core facility using the Trizol reagent method according to the manufacturer's directions (Invitrogen, Carlsbad, CA). The quality of total RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Columbia, MD). Reverse transcription was performed by using total RNA isolated from mouse tissues and processed with the Applied Biosystems (Foster City, CA) High-Capacity cDNA Archive kit first-strand synthesis system for RT-PCR according to the manufacturer's protocol. QRT-PCR was performed using the TaqMan assay system from Applied Biosystems. All PCR amplifications were carried out in triplicate on an ABI Prism® 7300 Sequence Detection System, using a fluorogenic 5' nuclease assay (TaqMan® probes).

The ΔC_t values for each sample were calculated by determining the difference in C_t values between the target genes [Hepcidin antimicrobial peptide (Hepc); Transmembrane protease, serine 6 (Tmprss6); Bone morphogenetic protein 6 (Bmp6); and Inhibitor of DNA binding 1 (Id1)] and the average of 3 endogenous control (housekeeping) genes [phosphoglycerate kinase 1 (Pgk1); Glyceraldehyde-3 phosphate dehydrogenase (Gapdh); Beta actin (Actb)]. The average of the ΔC_t values for each gene in each sample group was determined and then the average level of a gene relative to the housekeeping control genes in a group is given by $2^{-\text{average}\Delta Ct}$. The end points of the error bars about the average expression level relative to the control genes for each gene and group were determined by calculating the standard error of the mean (SEM) of the individual ΔC_t values for that gene and group, then calculating $2^{-\text{average}\Delta Ct + SEM}$ and $2^{-\text{average}\Delta Ct - SEM}$. Fold changes of gene expression between groups and corresponding p-values were calculated by using the $2^{-\Delta\Delta Ct}$ method [4].

Probes and primers were designed and synthesized by Applied Biosystems:

Assay ID	Gene Symbol	Gene Name -mouse
Mm00607939_s1	Actb	Beta actin
Mm99999915_g1	Gapdh	Gyceraldehyde-3-phosphate dehydrogenase
Mm00435617_m1	Pgk1	Phosphoglycerate kinase 1
Mm01332882_m1	Bmp6	Bone morphogenetic protein 6
Mm00519025_m1	Hamp	Hepcidin antimicrobial peptide
Mm00775963_g1	Id1	Inhibitor of DNA binding 1
Mm00551108_m1	Tmprss6	Transmembrane serine protease 6

Statistical Analyses: Statistical analyses were performed to study differences between two groups of mice, using Student's T-test with unequal variances. Genotype by age interactions were assessed using two way factorial ANOVA. Paired T-test was used to assess the longitudinal changes in hematologic parameters of Hepc KO vs. WT or IL-6 KO vs. WT mice. For cytokine analyses, the data were log transformed to approximate normality before analysis. Statistical significance was determined as $p \leq 0.05$.

Supplementary Results

Retired breeder female mice show evidence of increased inflammation when compared to virgin females.

After their purchase, female RB WT and RB IL-6 KO mice were aged in the JHU facility to 24 months of age as described in the main text. We assessed the complete blood count parameters of female RB WT and RB IL-6 KO mice between 9 and 13 months of age and compared these hematologic features to those of VF mice aged 9 to 13 months in the JHU facility (Table S1). Increased circulating neutrophils and platelets in the RB of both WT and IL-6 KO genotype indicated an increase in inflammation with breeding, but breeding status did not significantly impact on hemoglobin concentration at this time point (Table S1, $p= 0.80$ for genotype by breeder interaction).

IL-6 KO genotype modifies longitudinal change in hemoglobin in aged mice.

We followed erythrocyte parameters in the peripheral blood of an additional cohort of IL-6 KO mice aged in the UCLA facility longitudinally from 12-14 months to 20-25 months

of age (Table S2). At baseline, IL-6 KO mice had significantly increased erythrocytes and hemoglobin when compared to WT mice. Hemoglobin and erythrocyte number declined significantly in mice of both genotypes with age, but the magnitude of the decline in hemoglobin was significantly less for IL-6 KO mice than for WT mice, sparing hemoglobin in the aged IL-6 KO mice (Table S2, p=0.016 for genotype by age interaction). The difference in hemoglobin concentration at baseline for 12-14 month old mice in the UCLA facility (Table S2) versus the JHU facility (Tables 1 and 2 of the main manuscript) may reflect an earlier decline in hemoglobin in WT animals in the UCLA facility than in the JHU facility. We expect this observation may be related to differences in housing conditions between facilities or to a "survival effect" in the JHU mice which were selected for survival to 24 months of age and had lower incidence of tumors.

Hepc KO genotype modifies longitudinal change in hemoglobin in aged male mice.

We followed erythrocyte parameters in the peripheral blood of male (Table S3) or female (Table S4) Hepc KO mice aged in the UCLA facility longitudinally from 12-14 months to 17-23 months of age. At baseline, Hepc KO male and female mice had significantly increased erythrocytes and hemoglobin when compared to WT mice. Hemoglobin concentration declined significantly in mice of both genotypes with age, but the magnitude of the decline in hemoglobin was significantly less for male Hepc KO mice than for male WT mice, sparing hemoglobin in the aged male Hepc KO mice (Table S3, p=0.006 for genotype by age interaction). We observed the same trend for female Hepc KO mice, but the effect of genotype on the decline in hemoglobin with age did not reach statistical significance.

Effect of breeder status on erythropoietic activity at 24 months

In the main text, we have indicated that erythrocyte number (7.83 ± 1.45 vs. 8.68 ± 0.99, p=0.050) and hemoglobin concentration (10.8 ± 1.8 vs. 12.0 ± 1.2, p=0.023) were significantly lower in 24 month old RB WT compared to 24 month old VF WT mice. We tested whether we could also identify differences in iron stores and erythropoietic activity between RB WT and VF WT mice (Table S5). We did not detect significant differences for inflammatory cytokines, serum iron and tissue iron concentrations, and measures of erythropoietic activity between these two groups. Thus, the data from aged RB WT and aged VF WT mice were pooled (Table 4 of the primary manuscript). It should be noted however, that due to the small sample size, the insignificant difference between the two groups could reflect insufficient power. Because there was no significant difference in hemoglobin concentration between IL-6 KO aged RB and aged IL-6 KO VF, data for these groups were also pooled. Tissue iron stores, the percentage of committed splenic erythroid progenitors, and reticulocyte numbers were not determined for aged VF IL-6 KO mice as presented in Table S5.

Analysis of liver gene expression of targets in the Hepcidin regulatory pathway

We have provided Ct values corresponding to samples for each gene assayed in each individual mouse (Table S6). Relative values are shown in Figure 1A of the main manuscript. All of the Ct values were above background (Ct <36).

Erythroid maturation in bone marrow and spleen

We assessed the erythropoietic activity of the spleen in young and aged WT mice. Figure S1 depicts the gating strategy for erythroid progenitors in the spleen and provides more detail regarding the representation of erythroid precursors throughout stages I through V. We used the same strategy to define erythroid progenitors and precursors in the bone marrow (Figure 2 of the main text). We found a relatively higher percentage of erythroblasts and reticulocytes (stages II and IV) in the spleens of aged mice. This observation is consistent with the increased spleen weight and the increased percentage of Ter+ splenocytes and suggests extramedullary erythropoiesis is occurring in aged mice.

Supplementary Discussion

Here we have provided further analysis of the differences in hematologic parameters measured in our mouse colonies which were housed in different facilities. These observations may implicate differences in diet, housing, specified pathogens, or the target age of analysis in the development of anemia with age. Because of these extensive, but incompletely understood, differences among various facilities and study designs, we chose to compare either longitudinal or cross-sectional erythrocyte parameters within the same colony and facility.

RB mice are a useful way to accelerate the accomplishment of an aging mouse study. However, we have observed significant differences in erythrocyte parameters of RB mice at 24 months of age when compared to VF mice at 24 months of age despite over a year of housing in the same facility. We found that RB mice have increased circulating neutrophils compared to VF at their baseline bleed between 9 and 13 months in our facility. We hypothesize this may be the result of tissue remodeling after the stress of birthing pups, and while it does not seem to have an immediate impact on hemoglobin concentration, breeding status does seem to negatively impact on hemoglobin concentration by 24 months when the WT RB are compared to WT VF mice. A previous analysis of hematopoiesis in aged C57BL/6 mice compared hematologic features of very old single housed mice to those of very old group housed mice and concluded that stress induced by group housing resulted in changes in hematopoiesis [5]. The stress associated with birthing pups may act in a similar manner, altering hematopoiesis in RB mice.

Despite these differences, we have provided evidence to support the hypothesis that C57BL/6 mice are an appropriate model to study the molecular pathogenesis of the anemia that develops with age. No matter which colony or facility, all aged mice demonstrated a significant decline in hemoglobin from their baseline.

Supplementary Tables

Table S1. Hematologic features of virgin female and retired breeder mice at 9-13 months of age (JHU facility).

Parameter[$]	9-13 m VF WT N=34	9-13 m RB WT N=20	9-13 m VF IL-6 KO N=15	9-13 m RB IL-6 KO N=30	Genotype x breeder p-value[&]
Neutrophils (K/μL)	1.48 ± 0.49	2.11 ± 0.94*	1.19 ± 0.48	1.78 ± 0.90^	0.88
Monocytes (K/μL)	0.40 ± 0.16	0.46 ± 0.21	0.43 ± 0.21	0.36 ± 0.12	0.07
Lymphocytes (K/μL)	5.53 ± 1.77	6.41 ± 2.56	5.38 ± 2.09	5.54 ± 1.54	0.38
Erythrocytes (M/μL)	9.99 ± 0.46	9.21 ± 0.96*	9.87 ± 0.44	9.64 ± 1.09	0.11
Hemoglobin (g/dL)	14.3 ± 0.8	13.8 ± 1.1	14.4 ± 0.44	14.1 ± 1.5	0.80
MCV (fL)	44.9 ± 2.2	44.2 ± 1.8	44.9 ± 1.5	44.2 ± 2.6	0.95
MCH (pg)	14.3 ± 0.7	15.1 ± 0.9*	14.6 ± 0.5	14.7 ± 0.8	0.02
RDW (%)	18.6 ± 0.6	19.3 ± 2.9	18.1 ± 0.9	17.7 ± 1.5	0.10
Platelets (K/μL)	904 ± 197	1150 ± 322*	843 ± 201	1086 ± 304^	0.98

[$]± Standard Deviation; VF (virgin female), RB (retired breeder); p≤0.05, two sample t-test with unequal variance, *VF WT vs. RB WT; ^VF IL-6 KO vs. RB IL-6 KO; [&]2-way factorial ANOVA

Table S2. Longitudinal analysis of hematologic parameters of IL-6 KO mice with age (UCLA facility).

Parameter[$]	13-14 m WT N=6F + 4M	21m WT N=6F + 4M	12-14m IL-6 KO N=13F + 11M	20-25m IL-6 KO N=10F + 11M	Genotype x Age p-value[&]
Erythrocytes (M/μL)	9.01 ± 0.48	7.65 ± 0.91*	9.82 ± 0.65[#]	7.92 ± 0.63^	0.137
Hemoglobin (g/dL)	12.1 ± 0.4	8.0 ± 1.3*	14.0 ± 0.7[#]	11.3 ± 1.0^	0.016
MCV (fL)	44.7 ± 1.8	43.5 ± 3.9	45.0 ± 1.6	44.6 ± 1.5	0.426
Platelets (K/μL)	1013 ± 243	1251 ± 494	1694 ± 538[#]	1814 ± 567	0.210

[$]± Standard Deviation; *p≤0.05, paired T test of difference by age (13-14m vs. 21m) within WT genotype group; [#]p≤0.05, two-sample T test of difference between genotypes within age group (13-14m WT vs. 12-14m IL-6 KO); ^p≤0.05, paired T test of difference by age (12-14m vs. 20-25m) within IL-6 KO genotype group; [&] two-sample T test of the difference score between young and old by genotype

Table S3. Longitudinal analysis of hematologic parameters of male Hepc KO mice with age (UCLA facility).

Parameter[$]	13-14 m WT N=4	21m WT N=4	12-13m Hepc KO N=10	17-23m Hepc KO N=10	Genotype x Age p-value[&]
Erythrocytes (M/μL)	9.24 ± 0.15	7.29 ± 0.63*	10.22 ± 0.29[#]	9.29 ± 1.37	0.079
Hemoglobin (g/dL)	12.0 ± 0.4	7.4 ± 1.1*	14.3 ± 0.4[#]	13.0 ± 0.8^	0.006
MCV (fL)	43.9 ± 2.2	41.5 ± 0.85	47.8 ± 1.2[#]	46.4 ± 2.1	0.411
Platelets (K/μL)	1243 ± 197	1528 ± 652	1338 ± 149	1158 ± 209^	0.266

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

Table S4. Longitudinal analysis of hematologic parameters of female Hepc KO mice with age (UCLA facility).

Parameter[$]	13-14 m WT N=6	21m WT N=6	12-13m Hepc KO N=11	17-23m Hepc KO N=11	Genotype x Age p-value[&]
Erythrocytes (M/μL)	8.85 ± 0.58	7.90 ± 1.04	9.94 ± 0.66[#]	9.08 ± 2.04	0.900
Hemoglobin (g/dL)	12.2 ± 0.4	8.5 ± 1.3*	15.4 ± 0.8[#]	12.6 ± 2.2^	0.277
MCV (fL)	45.3 ± 1.4	44.8 ± 4.6	48.7 ± 3.2[#]	48.2 ± 4.8	0.999
Platelets (K/μL)	860 ± 113	1067 ± 288	968 ± 211	866 ± 270	0.132

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

Table S5. Measures of erythropoietic activity in virgin females and retired breeders at 24 months of age (JHU facility).

Parameter	24m VF WT N≥11	24m RB WT N≥ 8	p-value[&]	24m VF IL-6 KO N≥4	24m RB IL-6 KO N≥4	p-value[&]
IL6 (pg/mL)[‡]	72.7 ± 36.6	37.8 ± 64.4	NS	NA	NA	NA
IFNγ (pg/mL) [‡]	1.68 ± 1.30	1.34 ± 2.64	NS	2.05 ± 1.92	0.48 ± 39.15	NS
Liver iron (mcg/g) [$]	224 ± 84	256 ± 97	NS	ND	318 ± 94	ND
Spleen iron (mcg/g) [$]	2085 ± 1146	1553 ± 693	NS	ND	1617 ± 775	ND
Spleen weight (mg) [$]	156.6 ± 60.7	172.2 ± 88.1	NS	90.3 ± 25.7	64.6 ± 13.5	NS
Iron per spleen (mg) [$]	305 ± 161	225 ± 120	NS	ND	102 ± 48	ND
Serum Iron (mcg/dL) [$]	132 ± 31	113 ± 46	NS	131 ± 14	133 ± 34	NS
Ter+ Marrow (%)[$]	54.5 ± 9.5	52.1 ± 12.7	NS	58.6 ± 3.6	62.1 ± 7.8	NS
Ter+ Splenocytes (%)[$]	73.9 ± 11.1	74.7 ± 5.0	NS	ND	53.4 ± 10.6	ND
Retics (M/μL) [$]	0.337 ± 0.115	0.427 ± 0.285	NS	ND	0.245 ± 0.034	ND
Retics (%)[$]	4.20 ± 2.21	6.68 ± 6.89	NS	ND	2.67 ± 0.33	ND
Epo (pg/mL)[§]	0 (0-660)	10 (0-577)	NS	0 (0-131)	0 (0-115)	NS
Creatinine (mg/dL)[$]	0.27 ± 0.07	0.31 ± 0.04	NS	0.30 ± 0.08	0.23 ± 0.10	NS

[‡]Geometric mean ± geometric standard deviation, with corresponding tests performed on the log values of the parameter; [$]± Standard Deviation;

[&]2-way factorial ANOVA; [§]median and (range). VF (virgin female), RB (retired breeder), NA (not applicable), NS (not significant), ND (not determined)

Table S6. Original Ct values for liver gene expression analyses

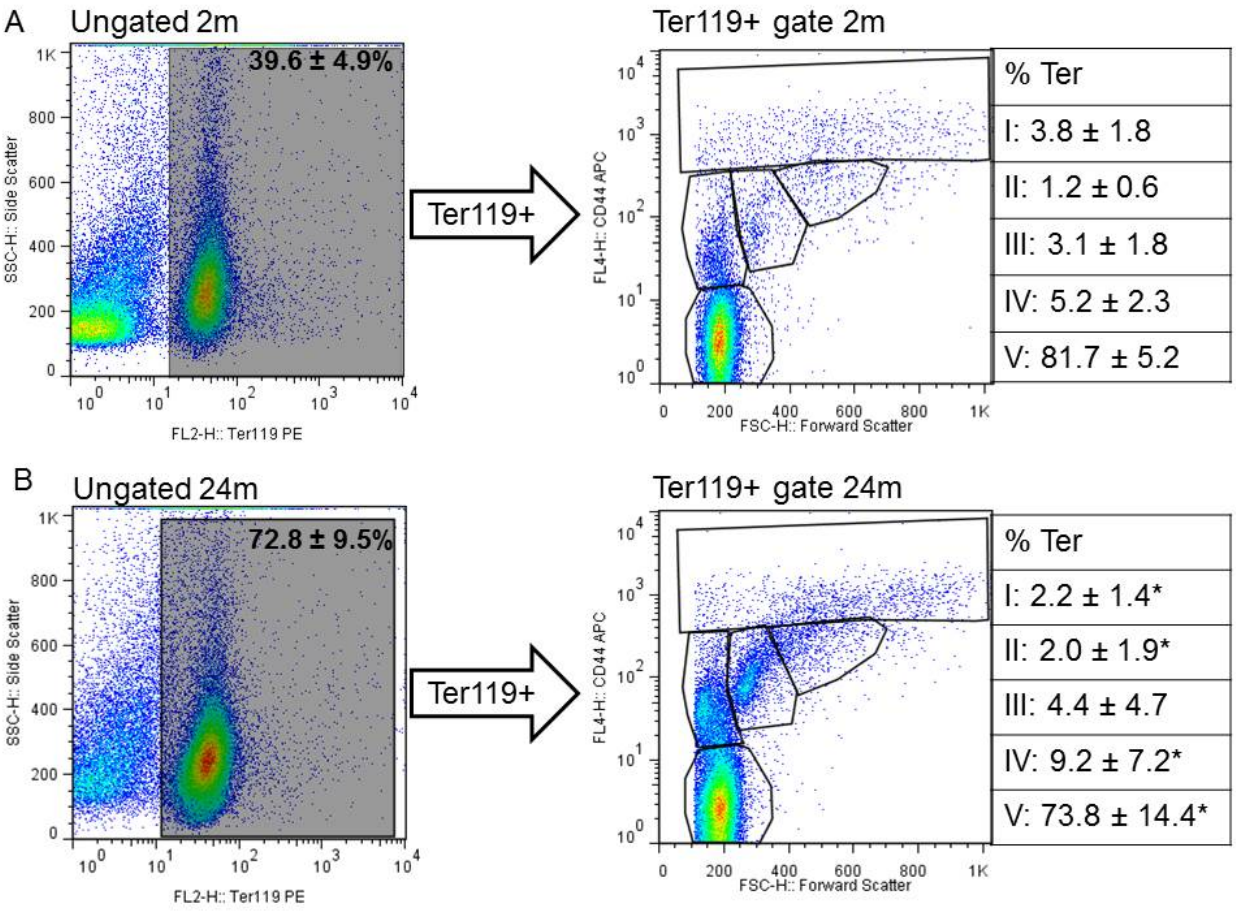
	IL-6 KO 24 months			C57BL/6 24 months											C57BL/6 2 months								
Bmp6	29.4	30.1	31.6	29.4	29.4	31.9	31.2	30.7	31.7	32.0	31.2	30.1	29.9	30.6	30.2	30.6	31.2	29.6	31.1	30.9	29.5	29.8	30.3
Bmp6	30.7	29.4	31.4	30.2	29.3	30.4	32.5	31.5	30.2	30.9	32.3	30.3	31.3	29.6	31.7	30.2	31.7	29.4	31.1	30.6	29.4	30.2	29.5
Bmp6	29.6	29.9	31.8	29.1	29.9	32.0	31.3	29.5	29.5	31.8	31.0	30.2	30.4	30.2	31.6	30.2	31.3	30.2	31.2	30.7	30.0	30.3	29.7
Hamp	21.8	20.3	22.0	20.0	19.8	20.0	20.4	20.0	20.2	21.1	20.3	21.0	19.6	20.0	21.4	21.7	21.0	20.4	21.1	20.4	20.1	20.4	21.5
Hamp	20.8	21.5	23.8	20.8	19.4	19.8	21.0	21.7	22.5	20.2	20.5	21.0	19.6	20.8	21.3	22.6	22.5	19.2	23.2	20.6	20.8	19.6	20.9
Hamp	20.2	20.7	22.2	20.1	20.2	19.8	19.9	19.5	19.6	20.7	19.3	20.6	19.4	19.5	21.5	21.3	21.2	19.6	21.7	20.6	20.1	19.9	20.3
Id1	27.0	27.3	28.2	26.8	26.0	25.9	27.3	25.8	27.3	26.7	25.6	27.1	26.9	26.4	28.6	27.1	28.2	27.6	29.2	28.2	27.9	27.4	26.8
Id1	25.8	28.7	28.2	26.5	26.1	25.8	28.5	26.2	27.6	26.7	25.5	28.6	26.7	26.1	28.7	27.9	28.0	27.3	29.1	28.3	27.7	27.7	26.4
Id1	25.7	27.3	28.0	27.2	26.1	27.4	27.6	26.3	28.1	26.2	25.5	27.0	27.1	27.0	28.4	27.3	28.1	27.8	29.3	28.4	28.1	27.3	26.6
Tmprss6	24.9	26.0	26.3	26.5	26.0	26.5	24.6	26.3	25.4	25.5	24.9	25.1	26.5	26.3	25.7	25.9	27.7	24.6	27.0	26.1	25.6	25.3	24.5
Tmprss6	24.9	25.1	26.9	26.2	24.8	26.4	25.8	26.2	24.2	25.4	25.6	25.3	26.9	25.3	26.0	25.4	27.1	24.7	26.6	26.2	25.3	25.4	24.8
Tmprss6	26.0	25.7	26.6	26.1	24.5	25.6	25.0	26.2	24.2	25.7	25.0	25.1	26.9	25.7	26.0	25.3	27.7	24.7	26.6	26.3	25.3	25.4	24.7
Actb	23.1	22.3	22.4	22.6	23.5	23.6	24.6	22.2	23.2	22.2	23.0	22.2	24.3	22.8	22.5	22.2	24.0	21.8	24.1	22.1	21.6	21.0	21.5
Actb	21.2	22.5	23.5	22.8	22.7	24.6	24.4	21.0	23.3	23.5	22.5	22.9	24.4	20.8	24.0	23.2	24.6	21.6	23.2	22.3	21.4	22.4	22.1
Actb	21.6	21.4	24.5	23.1	23.4	23.4	24.1	22.5	23.2	22.7	22.5	21.8	24.3	22.1	23.7	22.5	24.1	21.6	23.8	22.2	21.5	22.4	22.2
Gapdh	20.0	21.3	22.0	22.8	20.5	22.2	22.8	20.0	21.8	20.3	22.0	20.3	22.0	20.0	21.3	21.2	21.9	20.5	20.7	20.9	20.6	21.3	22.3
Gapdh	20.8	21.0	22.4	22.0	20.9	22.3	22.2	21.2	21.1	21.3	20.2	19.9	21.3	19.8	21.9	20.3	21.9	20.3	22.3	21.2	20.3	21.0	20.8
Gapdh	20.2	20.3	23.7	22.1	21.3	22.5	21.4	20.3	21.8	20.7	20.0	20.5	21.0	20.4	22.0	20.8	21.8	20.5	21.9	20.9	20.2	21.0	20.3
Pgk1	24.6	25.0	26.1	24.6	23.6	25.0	25.3	25.2	23.2	25.1	24.5	24.2	25.1	24.1	24.3	25.0	25.2	23.7	25.1	23.9	23.2	24.6	24.1
Pgk1	24.1	24.6	25.7	24.2	24.6	25.0	24.2	23.3	23.9	24.6	24.8	24.4	24.7	23.8	24.4	26.2	26.1	24.2	26.1	23.8	24.2	25.3	23.6
Pgk1	24.8	25.1	26.0	23.4	23.1	25.3	24.3	23.5	24.7	25.2	24.2	24.3	24.6	24.4	24.3	24.0	24.8	24.6	25.6	24.9	24.5	23.9	23.3

Supplementary Figure Legends

Figure S1. Gating strategy for splenic erythroid progenitors. Total splenocytes were stained with Ter119 and CD44. Ungated cells were selected for Ter119 positivity (left panels, shaded region). Ter+ cells were then viewed according to size (forward scatter) and CD44 expression (right panels). The same gating strategy was used for bone marrow erythroid progenitors described in Table 4 and Figure 2 of the main text. The frequency of erythroid progenitors and precursors in the spleen of 2 month old wild type C57BL/6 mice (A) was only 39.6 ± 4.9% (mean ± standard deviation), while the frequency of erythroid progenitors and precursors in the marrow of 24 month old wild type C57BL/6 mice was 72.8 ± 9.5% (B). The percentage of Ter+ cells in each stage of erythroid maturation (I-V) is represented numerically (mean ± standard deviation). Significance $p \leq 0.05$ is indicated with an asterisk and was determined by Student's t-test with unequal variance between groups.

Supplementary Figures

Figure S1. Gating strategy and frequency of splenic erythroid progenitors.



Supplementary References

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