Late stage erythroid precursor production is impaired in mice with chronic inflammation

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ABSTRACT

Background

We and others have shown previously that over-expression of hepcidin antimicrobial peptide, independently of inflammation, induces several features of anemia of inflammation and chronic disease, including hypoferremia, sequestration of iron stores and iron-restricted erythropoiesis. Because the iron-restricted erythropoiesis evident in hepcidin transgenic mice differs from the normocytic, normochromic anemia most often observed in anemia of inflammation, we tested the hypothesis that chronic inflammation may contribute additional features to anemia of inflammation which continue to impair erythropoiesis following the acute phase of inflammation in which hepcidin is active.

Design and Methods

We compared erythropoiesis and iron handling in mice with turpentine-induced sterile abscesses with erythropoiesis and iron handling in hepcidin transgenic mice. We compared erythrocyte indices, expression of genes in the hepcidin regulatory pathway, tissue iron distribution, expression of heme and iron transport genes in splenic macrophages, the phenotype of erythroid maturation and chloromethyl dichlorodihydrofluorescein diacetate, acetyl ester fluorescence.

Results

Mice with sterile abscesses exhibited an intense, acute inflammatory phase followed by a mild to moderate chronic inflammatory phase. We found that erythrocytes in mice with sterile abscesses were normocytic and normochromic in contrast to those in hepcidin transgenic mice. We also observed that although hypoferremia resolved in the late phases of inflammation, erythropoiesis remained suppressed, with evidence of inefficient maturation of erythroid precursors in the bone marrow of mice with sterile abscesses. Finally, we observed increased oxidative stress in erythroid progenitors and circulating erythrocytes of mice with sterile abscesses which was not evident in hepcidin transgenic mice.

Conclusions

Our results suggest that chronic inflammation inhibits late stages of erythroid production in the turpentine-induced sterile abscess model and induces features of impaired erythropoiesis which are distinct from those in hepcidin transgenic mice.

Key words: anemia, inflammation, erythroid precursor, mouse models.

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Introduction

Anemia of inflammation and chronic disease (AICD) is a collective term which describes any anemia in the setting of an inflammatory state. Classically, AICD has been observed in the context of infection by various pathogens. It is now commonly recognized in patients with autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus, but has more recently also been associated with chronic disease states such as chronic kidney disease, chronic heart failure, and aging, as reviewed by Roy.[1]

The clinical and molecular features of this mild anemia vary depending on the underlying inflammatory insult or disease, but commonly include iron sequestration, impaired erythropoietin production, impaired marrow response to available erythropoietin, and decreased erythrocyte life span.[1] Hepcidin antimicrobial peptide (hepcidin), a potent regulator of iron handling[2] that is induced by inflammation,[3-5] acts as the primary molecular mediator of the hypoferremia associated with the acute phase response. We have previously shown in humans[6] and mice[7] that over-expression of hepcidin results in iron sequestration and microcytic, hypochromic anemia.

In response to inflammation, hepcidin gene transcription is primarily induced through interleukin-6 (for clarity, abbreviated as "IL-6" throughout for both the mouse and human forms).[4] Increased hepcidin can be detected in the serum within 5 h of an IL-6 infusion in humans.[4] Hepcidin levels are elevated in the urine and serum of patients with acute infection,[8] multiple myeloma[9] and chronic kidney disease.[9] Given its role in iron sequestration in numerous disease states, hepcidin is a popular target for new therapeutics designed to treat AICD.

While increased expression of hepcidin in disease states is consistent with its hypothesized role in AICD, elevated hepcidin does not seem to explain the full picture of anemia in all chronic disease states. The anemia we observed in transgenic mice over-expressing hepcidin from a tetracycline-regulated promoter (hepcidin Tg+ mice)[7] is more consistent with the hypochromic, microcytic anemia observed in iron-deficiency anemia or iron-refractory, iron-deficiency anemia (IRIDA)[10] than with the normocytic, normochromic anemia often associated with AICD. While hepcidin is clearly essential to hypoferremia associated with the AICD phenotype, other mechanisms distinct from iron handling, including insufficient production of erythropoietin, inadequate erythroid progenitor response to erythropoietin, inefficient maturation of erythroid precursors, and decreased erythrocyte life span may also contribute importantly to anemia in some disease states such as chronic kidney disease, rheumatoid arthritis, and aging.[1]

To understand the molecular regulation of erythropoiesis in the setting of chronic inflammation better, we employed the turpentine-induced sterile abscess mouse model, a common rodent model of AICD.[3,11-22] In this model, hepcidin expression increases within 16 h of turpentine injection.[3] While hepcidin-mediated sequestration of iron stores would be expected to result in a microcytic, hypochromic anemia, similar to that in hepcidin Tg+[7] or the *Transmembrane serine protease 6* (*Tmprss6*) mutant, *mask* mouse,[23] the anemia in mice with chronic turpentine-induced sterile abscesses is normocytic with a reduced number of erythrocytes.[3] Recently, our colleagues demonstrated that inhibition of hepcidin expression with LDN-193189, a bone morphogenetic protein receptor inhibitor, significantly increased, but did not normalize, hemoglobin concentration in this model.[21] These data suggest mechanisms independent of hepcidin may further modulate hemoglobin concentration and erythrocyte number in this model.

To gain additional insight into the molecular regulation of erythropoiesis in response to inflammation, we compared the phenotype of the turpentine-induced sterile abscess model with that of the hepcidin Tg+ mouse. We assessed inflammatory markers, red blood cell features and the availability of iron stores in both of these models. Additionally, we investigated erythrocyte survival, maturation of erythroid precursors, and measures of reactive oxygen species which have not been thoroughly investigated in mice with turpentine-induced sterile abscesses or in hepcidin transgenic mice.

Design and Methods

Animal care

The Johns Hopkins University Animal Care and Use Committee approved all procedures involving mice. Mice were maintained on the 2018SX Teklad Global 18% Protein Extruded Rodent Diet (Harlan Teklad, Madison, WI, USA) which contains 225 parts per million (ppm) iron.

Hepcidin over-expressing transgenic mice

The generation of mice over-expressing the *Hepcidin* transgene under the control of the tetracycline regulatory element (TRE) has been previously described.[7] These mice were initially described on a mixed genetic background. We have since backcrossed both the Tg(tTALAP)5Uh[24] and the Tg(TRE.mhepcidin1) lines ten generations onto the C57BL/6 background. All hepcidin Tg+ mice described in this manuscript were 8- to 10-week old females.

Turpentine-induced sterile abscess

Chronic inflammation in female C57BL/6 mice was induced using the turpentine oil sterile abscess model which has been described previously.[3]

Enzyme-linked immunosorbent assays

Mouse pro-inflammatory cytokines [interferon gamma (IFNγ); IL-1β; IL-10; IL-12p70; IL-6; keratinocyte-derived cytokine; tumor necrosis factor alpha (TNFα)] were determined by multiplex analysis (Meso Scale Discovery, Gaithersburg, MD, USA). Anti-mouse erythropoietin and anti-mouse IL-6 enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The Wilcoxon Mann-Whitney non-parametric test was used to assess the significance of increased cytokine concentrations between the groups. To adjust for multiple comparisons, the level of statistical significance was chosen to be $P \leq 0.005$ for pro-inflammatory cytokines determined by multiplex analysis and $P < 0.02$ for erythropoietin determined by single-plex analysis.

Complete blood count

The complete blood count of whole blood samples was analyzed using a Hemavet 950 instrument (Drew Scientific, Waterbury, CT, USA). For statistical analysis of the complete blood count data, we used ANOVA for comparisons of measures between the C57BL/6 control, turpentine-induced abscess, and hepcidin Tg+ groups. Tests for difference between groups were

based on Bonferroni's multiple comparison test. The level of statistical significance was chosen to be $P \leq 0.05$.

Analysis of iron stores

Serum iron analysis was performed according to the manufacturer's directions using the Ferene Serum Iron/UIBC kit (Thermo Scientific, Fremont, CA, USA) for at least five mice in each group. We used the non-parametric Wilcoxon Mann-Whitney test for comparisons of measures between control, turpentine-induced abscess and hepcidin Tg+ mice. The level of statistical significance was chosen to be $P \leq 0.01$. Non-heme tissue iron was analyzed as previously described[7] in at least 14 mice in each group. Univariate ANOVA was used for comparisons of measures between control, turpentine-induced abscess and hepcidin Tg+ mice. Tests for differences between groups were adjusted for multiple comparisons with either Bonferroni's or Scheffe's multiple comparison tests. The level of statistical significance was chosen to be $P \leq 0.05$.

Gene expression

Splenocytes were washed and incubated at 4°C with rat anti-mouse F4/80 antibody (clone BM8, rat IgG2a; Invitrogen, Carlsbad, CA, USA), followed by incubation with goat anti-rat IgG microbeads (Miltenyi, Auburn, CA, USA). F4/80+ splenic macrophages were positively selected on MACS columns (Miltenyi). Total RNA was isolated from mouse livers or splenic macrophages by the Lowe Family Genomics Core facility using the Trizol reagent method according to the manufacturer's directions (Invitrogen). Quantitative real-time reverse transcriptase polymerase chain reactions (qRT-PCR) were performed using the TaqMan assay system from Applied Biosystems. All PCR amplifications were carried out in duplicate on an ABI Prism® 7300 Sequence Detection System, using a fluorogenic 5' nuclease assay (TaqMan® probes). Fold change values were obtained by computing $2^{-(\text{averageΔΔCt})}$ for genes in "trial" relative to control samples. The end-points of error bars were at the fold changes $2^{-(\text{averageΔΔCt}\pm\text{SEM})}$ where SEM is the standard error of the mean for $\Delta\Delta C_t$ calculated from the ΔC_t values from samples with four to eight mice in each group.

Flow cytometry and oxidative stress

Erythroid maturation was determined essentially as described elsewhere.[25,26] Flow cytometry data were analyzed with Flow Jo software (Tree Star, Ashland, OR, USA). To test for differences between the control, turpentine-induced abscess, and hepcidin Tg+ groups, we used ANOVA with Bonferroni's multiple comparison test. Analyses using Scheffe's test yielded similar results. The level of statistical significance level was chosen to be $P \leq 0.05$.

To quantify the presence of reactive oxygen species (ROS), dispersed bone marrow or whole peripheral blood was incubated for 30 min at 37°C with 4 μM chloromethyl dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen), a general ROS indicator. To test for differences between control and anemic groups, we used the non-parametric Wilcoxon Mann-Whitney test for two-sample comparisons of median fluorescence intensity (MFI) from samples run on the same day. We set the level of statistical significance at $P \leq 0.05$.

Further details on the materials and methods used in this study are provided in the *Online Supplementary Design and Methods*.

Results

Early and late inflammatory phases of mice with sterile abscesses

To investigate the molecular mechanisms driving anemia associated with chronic inflammation in mammals, we compared the phenotype of untreated C57BL/6 mice to that of two established mouse models of anemia of

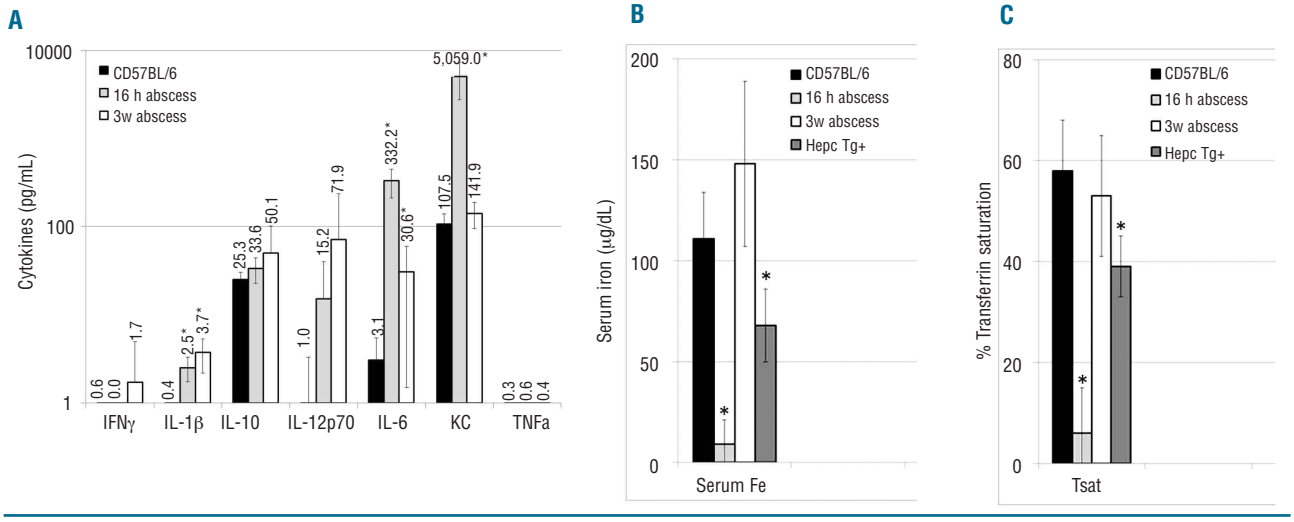

Figure 1. Acute and chronic inflammation in mice with sterile abscesses. (A) We assessed concentrations of inflammatory cytokines in the serum of mice after 16-24 h of sterile abscesses (gray bars, n=5 mice) and after 3 weeks of sterile abscesses (white bars, n=8 mice). The control group is represented by black bars (n=8 mice). We found that the concentration of IL-1β, IL-6 and keratinocyte-derived cytokine (KC) were all significantly elevated during the acute phase 16-24 h after turpentine injection. By 3 weeks, the concentration of these inflammatory cytokines had declined and only IL-1β and IL-6 remained significantly elevated. Bars represent the average concentration for each group. Error bars indicate the standard deviation. Significance $P \leq 0.005$ is indicated with an asterisk. (B) Serum iron concentration decreased significantly from 111± 23 μg/dL in control animals to 9±11 μg/dL 16-24 h after turpentine injection (gray bars, n=5 mice, $P=0.006$). After 3 weeks of abscesses (white bars, n=6 mice) the serum iron concentration of 148±41 μg/dL was not significantly different from that in untreated controls (black bars, n=7 mice). Serum iron was significantly decreased in hepcidin (Hepc) Tg+ mice at 68±18 μg/dL (dark gray bars, n=6 mice, $P=0.008$). Significance $P \leq 0.01$ is indicated with an asterisk. (C) Transferrin saturation followed a similar trend to that of serum iron. Significance $P \leq 0.01$ is indicated with an asterisk.

inflammation. The first model is the turpentine-induced sterile abscess model,[3,11-22] and the other is the hepcidin over-expressing transgenic (Tg+) mouse, which expresses hepcidin from a tetracycline-regulated promoter.[7] All mice were 8- to 10-week old female C57BL/6 mice.

To characterize the inflammatory state of mice with sterile abscesses, we assessed the expression of a panel of key inflammatory cytokines 16-24 h after the first turpentine injection and at 3 weeks (1 week after the third weekly turpentine injection). We found that the levels of IL-1β, IL-6, and keratinocyte-derived cytokine were significantly increased 16-24 h after turpentine injection (Figure 1A, $P<0.005$ for each, error bars indicate the standard deviation, note log scale). After 3 weeks, the levels of IL-1β and IL-6 remained statistically raised (Figure 1A, $P=0.001$ for both), consistent with this tissue injury model.[22] Increased IL-6 expression was also confirmed by single-plex ELISA (*data not shown*). The magnitude of the increase in IL-6 was nearly 100-fold greater than that in the control mice at the initial assessment 16-24 h after turpentine injection. However, IL-6 levels declined from this acute inflammatory state to remain 10-fold above control after 3 weeks. This time-course analysis did not assay IL-1β or TNFα at their peak expression, which we expect would occur prior to the IL-6 peak.

Analysis of the complete blood count confirmed that 8- to 10-week old female hepcidin Tg+ mice do not show evidence of elevated numbers of circulating inflammatory cells such as neutrophils or monocytes (Table 1). However, the numbers of circulating neutrophils and monocytes were significantly increased in mice with sterile abscesses ($P<0.001$ and $P<0.02$, respectively; Table 1). Consistent with an inflammatory process, platelet counts were also significantly increased in mice with sterile abscesses ($P<0.001$; Table 1). We did not observe a change in lymphocyte numbers in either model (Table 1). These results concerning the inflammatory cytokine profile and circulating inflammatory cells indicate that mice with sterile abscesses develop a durable, chronic inflammatory response, but that the cytokine response differs between the early (acute, 16-24 h) and late (chronic, 3 weeks) phases.

Early- and late-phase iron handling in mice with chronic sterile abscesses

To determine whether inflammation induced iron sequestration in mice with sterile abscesses, we measured serum iron concentration 16-24 h and 3 weeks after turpentine injection. Serum iron concentration and transferrin saturation were significantly decreased 16-24 h after turpentine injection ($P=0.006$ for both; Figure 1B,C). This observation is similar to those in previously published studies.[3] However, after 3 weeks of turpentine-induced abscesses, we found no significant difference in serum iron concentration or transferrin saturation between mice with abscesses and their controls (Figure 1 B,C). As expected, serum iron concentration was decreased in hepcidin Tg+ mice ($P=0.008$; Figure 1B).

Table 1. Mild hemoglobin decline in hepcidin Tg+ mice and mice with chronic sterile abscesses.

Parameter	C57BL/6 N=82	Abscess N=57	Hepcidin Tg+ N=44	ANOVA post-test
Weight (g)	17.1±1.8*	17.8±1.6	17.5±1.7	NS
Neutrophils (x10⁹/L)	0.55±0.36	1.43±1.72	0.59±0.29	B6 *vs.* SA <0.001 Tg+ *vs.* SA <0.001
Monocytes (x10⁹/L)	0.16±0.10	0.24±0.19	0.16±0.12	B6 *vs.* SA = 0.003 Tg+ *vs.* SA = 0.019
Platelets (x10⁹/L)	664±130	955±220	648±123	B6 *vs.* SA < 0.001 Tg+ *vs.* SA < 0.001
Lymphocytes (x10⁹/L)	2.98±1.66	2.87±1.69	2.91±1.28	NS
Hemoglobin (g/dL)	13.9±1.1	12.9±1.5	13.5±0.8	B6 *vs.* SA < 0.001 Tg+ *vs.* SA = 0.031 B6 *vs.* Tg+ = 0.160
Erythrocytes (x10¹²/L)	9.46±0.6	8.72±0.71	9.45±0.60	B6 *vs.* SA < 0.001 Tg+ *vs.* SA < 0.001
MCV (fL)	48.4±5.5	47.5±6.6	47.0±4.3	NS
MCH (pg)	14.7±0.9	14.8±1.1	14.3±0.7	B6 *vs.* Tg+ = 0.040 Tg+ *vs.* SA = 0.049
RDW (%)	17.0±0.8	18.9±0.8	17.6±1.2	B6 *vs.* SA < 0.001 B6 *vs.* Tg+ = 0.001 Tg+ *vs.* SA < 0.001
Reticulocytes (x10⁹/L)	280±48	336±84	271±82	B6 *vs.* SA = 0.010 Tg+ *vs.* SA = 0.013

*NS: no significant differences; *mean ± standard deviation. MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; RDW: red cell distribution width; B6: control mice; SA: mice with turpentine-induced sterile abscesses; Tg+: hepcidin over-expressing transgenic mice.*

Table 2. Hepcidin control of iron homeostasis in hepcidin Tg+ mice and mice with chronic sterile abscesses.

Parameter	C57BL/6 (N≥8)	Abscess (N≥6)	Hepcidin Tg+ (N≥8)	ANOVA post-test
Hepcidin (AU) hepatic mRNA	1.00	0.82 (0.44-1.56)*	1.23 (0.64-2.39)	B6 *vs.* SA = NS B6 *vs.* Tg+ = NS SA *vs.* Tg+ = NS
Tmprss6 (AU) hepatic mRNA	1.00	4.46 (1.64-12.15)	8.08 (6.25-10.46)	B6 *vs.* SA = 0.011 B6 *vs.* Tg+ <0.001 SA *vs.* Tg+ = NS
Bmp6 (AU) hepatic mRNA	1.00	1.22 (0.77-1.94)	0.71 (0.50-1.01)	B6 *vs.* SA = NS B6 *vs.* Tg+ = 0.058 SA *vs.* Tg+ = 0.016
Id1 (AU) hepatic mRNA	1.00	1.59 (0.89-2.85)	1.00 (0.66-1.52)	B6 *vs.* SA = NS B6 *vs.* Tg+ = NS SA *vs.* Tg+ = NS
Liver non-heme iron (mg/g)	85±19#	88±42	64±17	B6 *vs.* SA = NS B6 *vs.* Tg+ = 0.085 SA *vs.* Tg+ = 0.04
Spleen non-heme iron (mg/g)	684±191#	566±163	602±195	B6 *vs.* SA = NS B6 *vs.* Tg+ = NS SA *vs.* Tg+ = NS
Spleen:Liver non-heme iron	8.1±1.5#	7.3±2.9	9.5±2.1	B6 *vs.* SA = NS B6 *vs.* Tg+ = 0.169 SA *vs.* Tg+ = 0.020
Non-heme iron per spleen (mg)	42±13#	61±16	44±17	B6 *vs.* SA = 0.005 B6 *vs.* Tg+ = NS SA *vs.* Tg+ = 0.010
Spleen weight (mg)	64.7±18.6#	150.8±59.9	73.1±14.5	B6 *vs.* SA <0.0001 B6 *vs.* Tg+ = NS SA *vs.* Tg+ <0.0001
Serum erythropoietin (pg/mL)	47 (ND, 78)§	157 (ND, 610)	47 (ND, 287)	B6 *vs.* SA < 0.001 B6 *vs.* Tg+ = NS SA *vs.* Tg+ = 0.01

**fold change and 95% confidence interval of fold change; #standard deviation; ND: not detectable. §median (minimum value, maximum value). NS: not significant; B6: control mice; SA: mice with turpentine-induced sterile abscesses; Tg+: hepcidin over-expressing transgenic mice.*

Both IL-1β and IL-6, which were increased in mice with sterile abscesses (Figure 1A), mediate the acute phase response of the liver.[27] Both IL-1β and IL-6 have also been shown to induce expression of hepcidin.[4,5] Since the hypoferremia induced by sterile abscesses resolved by 3 weeks, we expected hepcidin expression might return to baseline as well, despite these low-grade pro-inflammatory signals. We, therefore, examined liver hepcidin expression by qRT-PCR (Table 2) at 3 weeks. We found considerable variability in hepcidin expression within groups. Neither mice with chronic sterile abscesses nor hepcidin Tg+ mice demonstrated significantly elevated hepcidin expression, although hepcidin Tg+ mice showed a trend toward elevated expression (Table 2; ≥6 mice per group). We have previously shown that hepcidin Tg+ mice increase hepcidin expression from the tetracycline-responsive promoter, but endogenous hepcidin levels are down-regulated to compensate for over-expression of the transgene.[7] We expect that the sum of these responses results in normalization of hepcidin expression in hepcidin Tg+ mice by 8 to 10 weeks of age.

To account for some of the variability in hepcidin expression and to assess whether the hepcidin promoter might receive competing regulatory signals, we assayed the liver mRNA expression of the negative hepcidin regulator, *transmembrane serine protease 6* (*Tmprss6*), and the positive hepcidin regulator, *bone morphogenetic protein 6* (*Bmp6*). *Tmprss6* mRNA was significantly increased in mice with abscesses (Table 2). If *Tmprss6* mRNA correlates with Tmprss6 functional activity, this would suggest that the hepcidin promoter may receive negative regulatory signals via Tmprss6, which would compete with the expected induction of hepcidin via IL-6 or IL-1β. *Tmprss6* mRNA was also significantly increased in the hepcidin Tg+ mice, again suggesting that the endogenous hepcidin promoter may receive negative regulatory signals which compensate for over-expression by the hepcidin transgene. This result is also consistent with the down-regulation of the endogenous hepcidin transcript that we have previously observed in hepcidin Tg+ mice.[7] *Bmp6* mRNA was significantly lower in hepcidin Tg+ mice than in mice with abscesses [consistent with reduced non-heme liver iron concentrations in hepcidin Tg+ mice (Table 2)], but expression of the *Bmp6* target gene, inhibitor of DNA binding 1 (*Id1*), was not significantly modified in either hepcidin Tg+ mice or mice with abscesses. These results suggest that increased expression of *Tmprss6* mRNA at 3 weeks may result in negative regulation of hepcidin expression, which may promote normalization of hepcidin expression in the later phase of inflammation in mice with sterile abscesses.

To determine whether the dynamic changes in hepcidin expression and serum iron concentration affected iron stores significantly, we assessed non-heme tissue iron stores in mice with sterile abscesses (Table 2). We observed no difference in total non-heme liver iron concentration in mice with abscesses compared to controls. While the concentration of non-heme iron in the spleens of mice with abscesses was not statistically different from that in controls, the significant increase in spleen size ($P<0.001$; Table 2) resulted in an absolute increase in the amount of iron stored in the spleens of mice with sterile abscesses ($P=0.005$, Table 2). Whether this increase in storage iron in the spleens of mice with sterile abscesses can be attributed to hepcidin-mediated iron sequestration early in the time-course or to physiological changes consistent with splenic extramedullary hematopoiesis (see *Online Supplementary Appendix*) is unclear.

Erythrocyte indices in mice with chronic sterile abscesses differ from those in hepcidin transgenic mice

Despite serum iron levels being normal in mice with sterile abscesses, hemoglobin and erythrocyte number were suppressed after 3 weeks of abscesses. The hemoglobin concentration was significantly lower in mice with abscesses than in control mice ($P<0.001$; Table 1). At 8 to 10 weeks of age, total hemoglobin concentration was only slightly decreased in hepcidin Tg+ mice, but mean cellular hemoglobin was significantly reduced ($P=0.04$; Table 1) which is consistent with iron-restricted erythropoiesis. Hepcidin Tg+ mice had a modest anemia and the "masked" phenotype of a hairless trunk[23] at 4 weeks (hemoglobin =11.3±0.9 g/dL in hepcidin transgenic mice *versus* 13.0±0.7 g/dL in 4-week old C57BL/6 mice, $P=0.004$), but the severity of the phenotype abates between 4 and 8 weeks on the C57BL/6 inbred background.

Importantly, the numbers of erythrocytes in mice with sterile abscesses, unlike in hepcidin Tg+ mice of any age, were significantly decreased compared to those in control mice ($P<0.001$; Table 1). This result was consistent with the previous characterization of anemia in mice with sterile abscesses,[3] but contrasts with the findings in hepcidin Tg+ mice, which are characterized by normal numbers of erythrocytes with less hemoglobin per cell (Table 1). In mice with sterile abscesses, the size and hemoglobin content of individual erythrocytes were indistinguishable from those in controls, but there were fewer erythrocytes. The red cell distribution width (RDW%) measures the variability of erythrocyte size and was significantly elevated in the mice with sterile abscesses and the hepcidin Tg+ mice (Table 1), a further indication that erythropoiesis is impaired in both models.

Despite a significant reduction in red blood cell counts, the numbers of reticulocytes were increased in mice with abscesses when compared to in hepcidin Tg+ mice or control mice ($P=0.01$ for both; Table 1). Furthermore, serum erythropoietin concentrations were higher in mice with abscesses than in hepcidin Tg+ mice or controls ($P\leq0.01$ for both; Table 2). Despite the significant expansion of extramedullary hematopoiesis, increased reticulocytes, and increased erythropoietin production, hemoglobin concentration and erythrocyte numbers did not exceed those in the hepcidin Tg+ mice, suggesting inefficiency in the development of erythroid precursors in mice with sterile abscesses. We believe that these data indicate that extramedullary hematopoiesis in the spleen was contributing to erythropoiesis and reticulocytosis in mice with sterile abscesses (see *Online Supplementary Appendix*), but was not sufficient to fully overcome the suppressive effects of chronic inflammation on erythrocyte production.

Erythroid precursor maturation is altered in mice with sterile abscesses

Based on the observation that mice with sterile abscesses have reduced numbers of circulating erythrocytes and increased reticulocytes, we assessed whether the survival of peripheral red blood cells was impaired. We found no reproducible difference in biotinylated peripheral blood

erythrocyte survival between control mice and mice with sterile abscesses (*data not shown*). This indicates that the low erythrocyte number in mice with sterile abscesses was not the result of more rapid removal of the cells from the circulation. In light of the reduced numbers of circulating erythrocytes, this result implies a deficiency in erythrocyte production.

To investigate the effects of inflammation on erythroid precursor production in mouse bone marrow, we used antibodies to assess stage-specific markers of erythropoiesis to analyze erythroid precursor maturation by flow cytometry. By analyzing erythroid maturation in total bone marrow, we aimed to assess possible effects of inflammation on all stages of erythroid development, as later stages of development are difficult to investigate with colony-forming assays. The cellularity of the bone marrow was not significantly different between control mice ($14.9\pm3.1\times10^6$ cells/femur, n=13) and mice with sterile abscesses ($12.8\pm3.5\times10^6$ cells/femur, n=14). Yet sterile abscesses induced the production of granulocytes (events with high forward and high side scatter, Figure 2B) in the marrow. These stain with the pan-leukocyte marker, CD45 (*data not shown*). Expansion of granulocytes without an increase in bone marrow cellularity implies an absolute reduction in erythroid precursors in the marrow (Figure 2B), consistent with the findings of a previous study.[19] However, we have observed this relative reduction in marrow erythroid precursors is partially compensated by the production of erythroid precursors in the spleen (*Online Supplementary Figure S1B*).

To assess the steady state maturation of erythroid precursors, we stained whole bone marrow from mice with abscesses or hepcidin Tg+ mice and their controls. We considered Ter119$^+$ events to be erythroid precursors because these cells possess a marker of terminal erythroid differentiation. We further discriminated erythroid precursors according to whether they expressed CD44[26,28] (Figure 2D-F) or CD71 (transferrin receptor, *data not shown*),[25,29] as previously described. Expression of both CD44 and CD71 declines as erythroid precursors reach later stages of differentiation. Using the CD44 marker for analysis, we found a significantly higher percentage of earlier Ter119$^+$ precursors ($P<0.005$; Figure 2E gates I, II and III) in mice with abscesses than in control mice or hepcidin Tg+ mice. It remains unclear whether this skewed distribution represents relative accumulation of early stage precursors (gates I, II and III), which would suggest a block in maturation, or whether the skewed distribution represents active removal of the terminal differentiation stages (IV and V). We did not find a statistically significant difference in the distribution of CD44$^+$/Ter119$^+$ precursors from the marrow of hepcidin Tg+ mice (Figure 2F) when compared to that in controls. When the analysis of erythroid maturation was restricted to hepcidin Tg+ mice with an average hemoglobin equivalent to that of the mice with abscesses (12.9±0.5 g/dL, n=6), we still did not find a significant difference in erythroid maturation between hepcidin Tg+ mice and controls. A similar loss in late stages of erythroid development in mice with abscesses, but not in hepcidin Tg+ mice, was observed using the CD71 marker for analy-

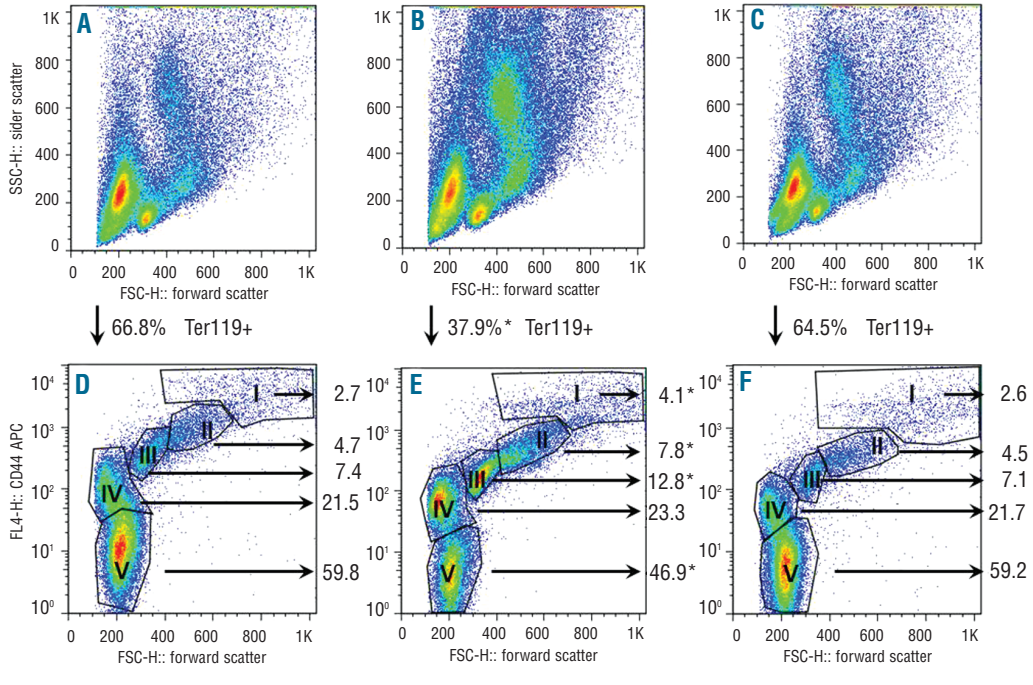

Figure 2. Erythroid maturation is impaired in mice with sterile abscesses. We assessed erythroid maturation in control mice (A and D), mice after 3 weeks of sterile abscesses (B and E), and hepcidin Tg+ mice (C and F). Granulocytes (events with high forward scatter and high side scatter) expand in mice with sterile abscesses (B). Total bone marrow cells were selected for Ter119 to define erythroid precursors (EP). Though the cellularity was similar in each of the groups, mice with sterile abscesses had a significantly reduced percentage of committed erythroid precursors (Ter119$^+$) (B). Ter119$^+$ precursors were further analyzed according to size (forward scatter) and CD44 expression which decreases as EP mature. We found a statistically significant increase in the percentage of early stage EP grouped in gates I, II, and III in mice with sterile abscesses (E). We also found a statistically significant decrease in the percentage of late stage EP grouped in gate V. Significance $P\leq0.05$ is indicated with an asterisk.

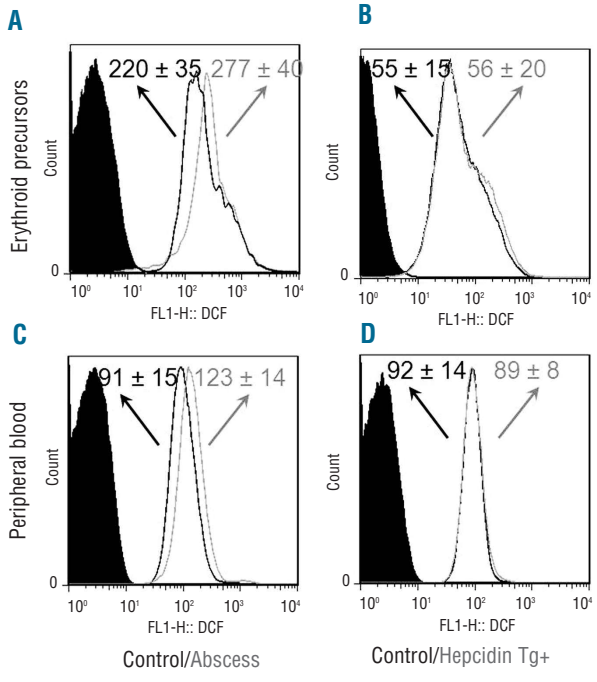

Figure 3. Median fluorescence intensity (MFI) of chloromethyl dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) in mouse bone marrow and peripheral blood. We assessed fluorescence of CM-H_2DCFDA, a sensor of reactive oxygen species (ROS) in erythroid precursors (A and B) or peripheral blood (C and D) of control mice (black lines, all panels), mice with sterile abscesses (gray lines, A and C), and hepcidin Tg+ mice [gray lines, panels (B) and (D)]. Unstained cells are indicated by the black filled histogram in each panel. Erythroid precursors were defined as CD45-negative events. We found a statistically significant increase in the MFI of erythroid precursors (*P*=0.04) and erythrocytes (*P*=0.02) in mice with sterile abscesses. We did not find a statistically significant increase in the MFI in hepcidin Tg+ mice.

sis (*data not shown*). We conclude that mice with sterile abscesses have impaired production or survival of late stages of erythroid progenitors because the maturation pattern of Ter119$^+$ precursors is skewed toward earlier (CD44$^+$/CD71$^+$) stages of development. We also conclude that, in contrast, hepcidin Tg+ mice have impaired hemoglobin production within each erythroid precursor because they have normal numbers of erythrocytes and erythroid precursor maturation does not differ from that of controls, based on this assay.

Increased oxidative stress in erythroid precursors of mice with sterile abscesses

We hypothesized that the skewed distribution of erythroid precursors in mice with abscesses (Figure 2E) might result from impaired erythroid maturation in late stages of development. Since later stages of erythroid development are predominantly characterized by hemoglobin production and preparation of the cells for their role as oxygen carriers, the proper handling of ROS is essential. ROS have been shown to increase in erythroid precursors when heme and globin chains are not sufficiently balanced[28,29] or when erythroid precursors are deficient in antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase.[30,31] We used a fluorescent dye, CM-H_2DCFDA, to assess whether erythroid precursors from the bone marrow of mice with sterile abscesses showed evidence of increased ROS. For these assays, we identified erythroid precursors as CD45-negative events.[32] We found that the median fluorescence intensity was higher for mice with abscesses than for controls (*P*=0.04; Figure 3A). In contrast, we found no significant difference in median fluorescence intensity of erythroid precursors between hepcidin Tg+ mice and controls (Figure 3B).

To assess whether the increased production of ROS in erythroid precursors from mice with sterile abscesses held for fully matured erythrocytes in circulation, we assessed CM-H_2DCFDA staining of peripheral blood. CM-H_2DCFDA median fluorescence intensity in the peripheral blood increased in mice with sterile abscesses (*P*=0.02; Figure 3C). Consistent with the results for hepcidin Tg+ erythroid precursors, we found no significant difference in CM-H_2DCFDA median fluorescence intensity in the peripheral blood of hepcidin Tg+ mice (Figure 3D). These results suggest that ROS are increased in erythroid progenitors and remain in circulating erythrocytes of mice with sterile abscesses.

Discussion

In this study we have demonstrated that mice with chronic inflammation resulting from sterile abscesses have impaired erythropoiesis that differs mechanistically from the iron-restricted erythropoiesis which develops in mice over-expressing a mouse hepcidin transgene. By comparing these models, we have provided *in vivo* evidence for pathogenic mechanisms that may impair erythropoiesis beyond the iron sequestration induced by hepcidin during the acute phase of inflammation in mice with sterile abscesses. Red blood cell indices, serum iron concentration and extramedullary hematopoiesis in the mice with sterile abscesses suggest mechanistic differences between the sterile abscess model and the hepcidin Tg+ model. Erythrocyte numbers fell significantly only in mice with abscesses. Hepcidin Tg+ mice have normal erythrocyte numbers and significantly decreased mean cell hemoglobin, while the mice with sterile abscesses have significantly reduced erythrocyte numbers and normal mean cell hemoglobin. This feature of the sterile abscess model is more akin to the classically described AICD than to the anemia in hepcidin Tg+ mice, which only exhibit iron-restricted erythropoiesis.

In the sterile abscess model, hypoferremia was restricted to the very early stages of inflammation despite a prolonged inflammatory response and lasting suppression of erythropoiesis. Serum iron concentration returned to normal in mice after 3 weeks of abscesses. The data presented here suggest that multiple pathways which influence serum iron concentration are simultaneously regulated. The data we have presented that support iron retention in splenic macrophages of mice with sterile abscesses include elevated IL-6, a known positive transcriptional regulator of hepcidin; reduced membrane-bound ferroportin in splenic macrophages (see *Online Supplementary Appendix*); and more total iron in the spleen. Despite these signals of iron sequestration, serum iron concentrations may normalize in mice with sterile abscesses because Tmprss6 eventually normalizes hepcidin expression, because impaired erythropoiesis may result in reduced clearance of transferrin-bound iron, or because extramedullary erythropoiesis in the spleen may uniquely support erythropoiesis such that

splenic erythroid progenitors can circumvent usual requirements for transferrin-bound iron.

These changes in iron handling that we observed in mice with sterile abscesses may be unique to the sterile abscess model, since hypoferremia is the most commonly reported feature of AICD. However, anemia can occur in the context of inflammation without hypoferremia. In a prospective study of all patients with anemia admitted to a county hospital, Cash and Sears found a significant number of patients with anemia and infections or inflammatory disease who should have been good candidates for AICD, but who did not fit the strict criteria for AICD.[33] Many of these patients had normal iron parameters. Thus, AICD may occur in the absence of long-term disturbances in iron cycling. Our data raise the possibility that reduced iron demand resulting from suppressed erythropoiesis may contribute to normal iron parameters in these individuals.

Importantly, we observed impaired maturation of erythroid precursors in the bone marrow of mice with sterile abscesses. We also observed a modest increase in CM-H_2DCFDA staining in the bone marrow erythroid precursors and peripheral blood of mice with abscesses, indicating some level of oxidative stress intrinsic to the red cell compartment. In contrast, erythropoiesis in hepcidin Tg+ mice seems to occur unimpeded, except for reduced hemoglobin production resulting from hepcidin-induced iron sequestration. These observations may imply an imbalance in heme and globin production or insufficient production of anti-oxidant enzymes in the surviving erythroid precursors from mice with sterile abscesses. Given the rapid clearance of malformed erythroid precursors, our assays might not be able to detect the cells that do not survive the "quality control" process of the central macrophages of erythroid islands in the bone marrow.[34]

Until the discovery of hepcidin, most investigation of AICD centered around the ability of individual cytokines to regulate proliferation and maturation of erythroid precursors *in vitro*.[35,36] Individual cytokines, such as IL-6, have also been correlated with the severity of the anemia in chronic disease states.[37-39] Colony-forming assays are robust assays of proliferative capacity, but they are not well suited for the analysis of the later stages of erythroid maturation. Late stages of erythroid maturation proceed independently of erythropoietin and after the majority of cell divisions have occurred. Colony-forming assays are also limited in that the *in vitro* microenvironment may not adequately reflect the erythroid developmental niche.

Our results provide *in vivo* evidence for impaired erythropoiesis at Ter119$^+$ stages of erythroid development, which we expect occur after the erythropoietin-dependent stages of erythroid development have been completed.[40] We hypothesize that this skewed distribution of erythroid-specific "immunophenotypic" markers in mice with sterile abscesses indicates impaired maturation of late stage precursors. Future studies should address the balance of heme and globin synthesis and anti-oxidant enzyme activity in erythroid precursors of mice with sterile abscesses and address whether these features are conserved in human erythroid progenitors in the context of inflammation. The impact of inflammation on late stages of mammalian erythroid development may suggest a need for novel, eyrthropoietin-independent clinical interventions. Therapies that affect the latest stages of erythroid development would be expected to complement the use of erythroid-stimulating agents, providing potentially safer and more cost-effective strategies for the treatment of AICD.

Authorship and Disclosures

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