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Extramedullary erythropoiesis in the adult liver requires BMP4/Smad5 dependent signaling

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Abstract

Objective—In mice, homeostatic erythropoiesis occurs primarily in the bone marrow. However, in response to acute anemia, BMP4 dependent stress erythropoiesis occurs in the adult spleen. BMP4 can also regulate stress erythropoiesis in the fetal liver. In humans, erythropoiesis occurs in the bone marrow. However, in certain pathological conditions, extramedullary erythropoiesis is observed where it can occur in several organs including the liver. Given these observations, we propose to investigate whether the BMP4 dependent stress erythropoiesis pathway can regulate extramedullary erythropoiesis in the livers of splenectomized mice.

Methods—Using splenectomized wildtype and *flexed-tail (f)* mice, which have a defect in BMP4 signaling, we compared their recovery from Phenylhydrazine induced hemolytic anemia and characterized the expansion of stress BFU-E in the livers of these mice during the recovery period.

Results—Our analysis indicates that in the absence of a spleen, stress erythropoiesis occurs in the murine liver. During the recovery, stress BFU-E are expanded in the livers of splenectomized mice in response to BMP4 expressed in the liver. *fff* mice, which exhibit a defect in splenic stress erythropoiesis do not compensate for this defect by up-regulating liver stress erythropoiesis. Furthermore, splenectomized *fff* mice exhibit a defect in liver stress erythropoiesis, which demonstrates a role for the BMP4 dependent stress erythropoiesis pathway in extra-medullary erythropoiesis in the adult liver.

Conclusions—Our data indicate that the BMP4 dependent stress erythropoiesis pathway regulates extra-medullary stress erythropoiesis, which occurs primarily in the murine spleen or in the case of splenectomized mice, in the adult liver.

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Keywords

Extra-medullary erythropoiesis; BMP4; Anemia

Introduction

In adults steady state erythropoiesis occurs in the bone marrow and is primarily homeostatic. The situation is dramatically different during embryonic development and in response to acute anemia. At these times stress erythropoiesis is induced to rapidly generate new erythrocytes to meet the increased need. It has long been suggested that fetal liver erythropoiesis and stress erythropoiesis may be regulated by similar mechanisms. These similarities are highlighted in mice mutant at the murine *flexed-tail* (*f*) locus. *f/f* mutant mice exhibit a severe fetal anemia early in development, which progressively improves such that by three weeks after birth the mice exhibit normal erythrocyte counts [1–6]. As adults, *f/f* mice exhibit normal steady state blood values and have numbers of CFU-E and BFU-E in their bone marrow that are comparable to wild type mice [7,8]. In contrast to their normal steady state erythropoiesis, *f/f* mice have severe defect in stress erythropoiesis. They exhibit a delayed recovery from acute anemia [7–9]. We have analyzed *f/f* mice and shown that the delay in recovery is caused by the delayed expansion of a specialized population of stress erythroid progenitors in the spleen, which we term, stress BFU-E [8]. We cloned the *f* locus and showed that *f/f* mice have mutation in *Smad5* [8,10], a transcription factor that functions downstream of the receptors for bone morphogenetic proteins 2, 4 and 7 (BMP2, 4 and 7) [11]. This mutation causes a tissue specific splicing defect that results in the production of a dominant negative *Smad5* protein in stress erythroid progenitors [8,10]. BMP4 expression is induced in the spleen just prior to the expansion of stress BFU-E [8]. Our analysis of the signals required for the expansion of stress BFU-E also demonstrated that BMP4 acts in concert with SCF and hypoxia to promote the proliferation and differentiation of stress BFU-E [12]. These data allowed us to develop a new model for stress erythropoiesis where stress erythroid progenitors resident in the spleen respond to BMP4, SCF and hypoxia dependent signals, differentiate into stress BFU-E, which rapidly proliferate and differentiate into new erythrocytes. Stress BFU-E are ideally suited to respond to acute anemia because they have a greater capacity to generate large numbers of new erythrocytes faster than steady state bone marrow BFU-E [8,12]. We have also shown that the anemia observed in *f/f* embryos early in fetal development is caused by a defect in stress erythropoiesis in the fetal liver. At a critical time in development (Embryonic day 13.5–14.5), BMP4 is expressed in the fetal liver, which promotes the rapid expansion of stress BFU-E. *f/f* embryos exhibit a delay in the expression of BMP4 and a delayed and diminished expansion of stress BFU-E [6]. These data demonstrate that adult stress erythropoiesis and fetal liver erythropoiesis are regulated by identical mechanisms.

Our analysis shows that the BMP4 dependent stress erythropoiesis pathway in mice functions in the fetal liver and the adult spleen [6,8]. In humans, the situation may be different. Certain pathological conditions can result in extra-medullary erythropoiesis in the liver, spleen and other organs [13–22]. Early work in the field showed that when mice were given repeated injections of phenylhydrazine (PHZ) to induce anemia, erythropoiesis was observed in the liver [23,24]. In this report, we investigate whether the BMP4 dependent stress erythropoiesis pathway functions in the adult liver during the recovery from acute anemia. We show that in splenectomized mice, stress erythropoiesis occurs in the liver and is characterized by the expansion of stress BFU-E. Furthermore, we show that splenectomized *f/f* mice exhibit a delayed and diminished expansion of stress BFU-E, which demonstrates that the BMP4 dependent stress erythropoiesis pathway is responsible for extra-medullary erythropoiesis in the adult liver as well as the spleen and fetal liver.

Methods and Materials

Mice

C57BL/6 and C57BL/6 splenectomized mice were obtained from Jackson Laboratory (Bar Harbor, ME). C57BL/6-*f/f* mice were bred in our colony. For splenectomy, C57BL/6-*f/f* mice were anesthetized with Ketamine/Xylazine (80 mg/kg; 10mg/kg) injected IP. An incision was made in the skin and body muscles. The spleen vessels were tied and the spleen was removed. The body wall incision was sutured and skin closed with wound clips. 7 days after surgery the wound clips were removed. Splenectomized mice were allowed to recover an addition 14 days before treatment with PHZ. Acute anemia was induced as previously described [8]. All procedures were approved by the IACUC of the Pennsylvania State University.

Analysis of stress BFU-E

Analysis of BFU-E in the bone marrow was done as previously described [6,8,12,25]. In short, cells were plated at the indicated concentrations in methylcellulose media (M3334, Stem Cell Technologies, Vancouver BC) supplemented with the indicated growth factors. Cells were grown at 37C for 5–7 days and BFU-E scored by acid benzidine staining [26]. For each mouse, the assays were done in triplicate. For the analysis of BFU-E in liver, single cell suspensions of liver cells were generated by treating liver tissue with Type I collagenase (440 ug/ml) (Worthington, Lakewood, NJ). Erythrocytes were lysed with cold 0.16M ammonium chloride (Sigma, St. Louis, MO). Mononuclear cells were isolated using a Nycoprep 1.077 step gradient (AXIS-SHIELD PoC AS, Oslo, Norway). BFU-E were assayed as described above [6,8,12, 25].

Analysis of BMP4 expression in the liver

Livers were harvested at the indicated times post PHZ induced anemia, fixed in 4% paraformaldehyde, and paraffin embedded tissue sections cut. BMP4 staining was done as described [6,8,25], except an HRP conjugated secondary antibody was used. HRP activity was detected using HRP detection kit (Pharmingen, San Diego, CA) according to manufacturer's instructions.

Statistical analysis

P values were calculated using Student's T test.

Results

Splenectomized control mice recover from PHZ induced anemia with different kinetics

Our previous work demonstrated that during the recovery from PHZ induced acute anemia, a specialized population of stress erythroid progenitors resident in the spleen are rapidly expanded in response to BMP4, SCF and hypoxia dependent signals[8,12]. Given that extra-medullary erythropoiesis is observed in several organs besides the spleen[27], we sought to determine whether this pathway was responsible for regulating stress erythropoiesis in organs other than the spleen. Splenectomized and control C57BL/6 mice were treated with a single dose of PHZ (100 mg/kg mouse) and the recovery of hematocrit was measured over an 8 day period. The data in figure 1 show that the ultimate recovery of splenectomized and control mice occurred by 8 days after treatment, however the kinetics of recovery was different in splenectomized mice. The hematocrits of control mice reached a nadir at 48 hours after PHZ treatment and by three days after treatment, their hematocrits started to increase. In contrast the hematocrits of the splenectomized mice reached a nadir between 4 and 5 days after treatment, but rapidly recovered such that the mice exhibited near normal hematocrits by day 8. These data show that the absence of a spleen changes the kinetics of recovery from acute

anemia and suggest splenectomized mice compensate for the loss of the stress erythropoiesis in the spleen.

The bone marrow does not expand progenitors to compensate for the loss of the spleen

Our previous analysis showed that the expansion of BFU-E in the bone marrow during the recovery from acute anemia was modest compared to the expansion observed in the spleen [8]. The similar overall recovery time and altered kinetics of recovery observed in splenectomized mice suggests that another organ may be compensating for the lack of splenic stress erythropoiesis. We tested whether the bone marrow exhibits an increased expansion of BFU-E in splenectomized mice following PHZ treatment. We did observe an increase in bone marrow BFU-E at 12 hours after PHZ treatment. However, at no other time points did we observe an increase in bone marrow BFU-E. Based on these data, we conclude that there was no overall increase in BFU-E that would suggest that the bone marrow compensated for the loss of splenic stress erythropoiesis (Figure 2A). Furthermore, we did not observe any increase in the number of BMP4 responsive stress BFU-E in the bone marrow beyond the small numbers present in control mice (data not shown).

Stress BFU-E expand in the liver of the splenectomized mice

Extra-medullary erythropoiesis has been observed in several organs in addition to the spleen, most prominently in the liver[27]. Furthermore, recent work from our lab has demonstrated that the BMP4 dependent stress erythropoiesis pathway plays a key role in regulating fetal liver erythropoiesis, which suggests that the liver has the potential to support stress erythropoiesis [6]. We tested whether BFU-E were present in the livers of splenectomized and control mice during the recovery from acute anemia. In Figure 2B we show that untreated splenectomized and control mice have few BFU-E in their livers. However by 3 days after PHZ treatment, BFU-E are significantly expanded in the livers of splenectomized mice (65 fold) and elevated BFU-E levels are maintained at 4 and 5 days post treatment. A smaller expansion is also observed in control mice (~20 fold at day 4), however, the expansion of BFU-E is far less in control mice when compared to splenectomized mice. This expansion of BFU-E correlates with the delayed kinetics and rapid recovery observed during days 6, 7 and 8 after PHZ treatment. Our previous work showed that the spleen expands a specialized stress erythroid progenitor, which we termed the stress BFU-E[8,12]. These progenitors are characterized by their ability to form BFU-E colonies in media containing only Epo. We tested whether the BFU-E expanded in the liver exhibited properties of stress BFU-E. When we plated liver mononuclear cells in methylcellulose media containing Epo alone, we observed the same expansion of BFU-E as was observed when cells were plated in media containing both Epo and IL-3 suggesting that most of the BFU-E in the liver are stress BFU-E. These data demonstrate that stress erythropoiesis in the liver leads to the expansion of stress BFU-E similar to that which was observed in the spleen.

Extra-medullary erythropoiesis in the livers of splenectomized mice was further demonstrated by histological analysis of liver sections. Staining of liver sections from untreated mice (splenectomized or control) revealed little evidence of erythropoiesis, which supports the idea that liver erythropoiesis is induced in response to acute anemia. However, beginning on day 4 after treatment, splenectomized mice exhibited numerous erythropoietic foci, which increased in size and number by day 6 after treatment and were still present at day 8 (Figure 3A-Top). In contrast, control livers exhibited few small foci at day 4, which failed to increase in size or number during the recovery. The lack of erythropoietic foci in the control mice is not surprising because control mice are already starting to recovery by 4 days post PHZ treatment and have near normal hematocrits by day 6. Spleen stress erythropoiesis in the control mice generates sufficient numbers of new erythrocytes to preclude the need for liver erythropoiesis.

The expression of BMP4 is expanded in the livers of splenectomized mice and correlates with erythropoiesis

The expansion of stress BFU-E in the spleen during the recovery from acute anemia is preceded by induction of BMP4 expression. We next tested whether BMP4 is expressed in the livers of splenectomized and control mice following treatment with PHZ. Immunohistochemistry staining of liver sections showed that BMP4 is constitutively expressed in the livers of both mice at all times during the recovery period. However, closer examination of the staining revealed a difference between the splenectomized and control livers. The functional unit of liver organization, the liver acinus, consists of adjacent classic lobules partially separated by distributing vessels from the portal triad (consisting of the portal vein, hepatic artery and bile duct(s)) and central veins at each pole (Figure 3B)[28]. Analysis of the oxygen tension in this functional unit has shown that the region immediately surrounding the central vein contains the lowest oxygen concentration (Figure 3B – Zone 3), while the region between triads contains the highest oxygen concentration (Figure 3B – Zone 1)[28–31]. BMP4 is expressed in zone 3 of untreated mice, which is consistent with our earlier findings that BMP4 is regulated by hypoxia[8]. During the recovery from acute anemia the expression of BMP4 expands to zones 1 and 2. In control mice this expansion is temporary such that BMP4 expression is exclusively in zone 3 by day 4. In splenectomized mice, however, the expansion of BMP4 expression persists until day 6, which correlates with the hematocrit recovery in splenectomized mice. Furthermore, erythropoietic foci are present in the regions expressing BMP4 (Figure 3C). BMP4 signaling leads to phosphorylation of Smads1, 5 and 8. When we stained liver sections with anti-phospho-Smad1, 5, 8, we observed that liver sections from splenectomized mice showed increased staining 24 hours after PHZ treatment when compared to sections from control livers (data not shown). These observations suggest that in the absence of a spleen the expression of BMP4 in the liver is expanded and persists so that expansion of stress BFU-E in the liver can compensate for the loss of the splenic stress erythroid response.

Splenectomized *f/f* mice exhibit a more severe defect in the recovery from acute anemia

Our analysis supports the hypothesis that in splenectomized mice, the BMP4 dependent stress erythropoiesis pathway is activated in the liver to compensate for the loss of the spleen response. To demonstrate that the stress erythroid progenitors in the liver required BMP4 signaling, we tested whether splenectomized *f/f* mice were able to expand stress BFU-E in the liver. *f/f* mice exhibit a delayed recovery from PHZ induced acute anemia, which resolves between day 10 and 11 after treatment[7–9,32]. This delay is further exacerbated in splenectomized *f/f* mice where the hematocrits of surviving mice reach a lower nadir when compared to *f/f* controls. Furthermore, by day 11 after treatment, the splenectomized *f/f* mice have not recovered to the same extent as controls (Figure 4A). We also observed greater mortality in the splenectomized *f/f* mice. In Figure 4B, we show that while 100% of the *f/f* control mice survived for 11 days after treatment, only 70% of the splenectomized mice survived. The most likely cause of death in splenectomized *f/f* mice is the severe anemia that developed following PHZ treatment.

Our previous work showed that the delayed recovery from acute anemia observed in *f/f* mice was a consequence of the delayed expansion of stress BFU-E in the spleen[8]. We next examined whether stress BFU-E were expanded in the livers of splenectomized *f/f* and whether the expansion of stress BFU-E was delayed. The data in Figure 4C demonstrates that the expansion of stress BFU-E is delayed.

Control splenectomized mice exhibited peak expansion of stress BFU-E 3 days after PHZ treatment (Figure 2B) while in splenectomized *f/f* mice the peak expansion of stress BFU-E is delayed until day 4 after treatment. In addition, the overall expansion of stress BFU-E was less than that at observed in control splenectomized mice (Figure 4C and compare with Figure 2B).

The delayed expansion correlates with a delayed and diminished appearance of erythropoietic foci in the livers of splenectomized *f/f* mice (Figure 3A-Bottom). However, when we examined the expression of BMP4 in the livers of *f/f* splenectomized and control *f/f* mice, we did not observe any differences in expression (Figure 3D). Although the expression was similar in the *f/f* and *f/f* splenectomized mice, it was distinct from the expression pattern observed in splenectomized and control C57BL/6 mice. BMP4 was widely expressed in untreated mice and expanded expression persisted longer during the recovery time. This observation is similar to what we observed in the adult spleen during the recovery from acute anemia[8]. We consistently observed constitutive expression of BMP4 in *f/f* mutant spleens, while expression in control spleens was induced only during recovery. Taken together these data demonstrate that the BMP4 dependent stress erythropoiesis pathway plays a key role in the development of extra-medullary erythropoiesis in the adult spleen and liver in response to acute anemia.

Discussion

The erythropoietic system has the capacity to respond to acute anemic stress. Previous work from our lab showed that the murine spleen provides a supportive microenvironment that promotes the expansion of a specialized population of stress erythroid progenitors[8,12,33]. Although the spleen is the primary site of stress erythropoiesis in the mouse, extra-medullary erythropoiesis has been observed in the liver[23,24,27]. Here our data show that in splenectomized mice, the BMP4 dependent stress erythropoiesis pathway functions in the adult liver. Early work in the field showed that erythropoiesis occurs in the liver at times of severe anemia. Our analysis extends these early observations to show that stress BFU-E expand in the liver in response to BMP4 dependent signals. BMP4 is expressed in the livers of untreated mice, however in response to PHZ induced anemia, BMP4 expression in the liver is increased and site of expression expands to fill nearly the entire liver functional unit. In control mice the increased expression of BMP4 is transient. In contrast, splenectomized mice maintain wide spread BMP4 expression. These data coupled with our observations that splenectomized *f/f* mice exhibit a defect in hepatic stress erythropoiesis demonstrate that the BMP4 dependent stress erythropoiesis pathway regulates hepatic extra medullary erythropoiesis.

Earlier studies on the response to PHZ induced anemia showed that erythropoiesis in the liver was observed in non-splenectomized mice [23,24]. These studies used a regimen of PHZ injection that included multiple injections, which resulted in a more prolonged and severe anemia than that observed using our single injection protocol. Despite the difference in the methodology, we did observe a low level expansion of stress BFU-E in the livers of control mice, which was not as robust as that observed in the splenectomized mice. BMP4 expression is transiently up regulated through out the liver functional unit at this time. Despite the expansion of stress BFU-E in the control livers, we did not observe significant numbers of erythropoietic foci in these livers. The foci represent the differentiated progeny of the stress BFU-E. These data suggest that although stress BFU-E expand in the livers of control mice during recovery, they do not differentiate. Our previous work has demonstrated that the maximal expansion and differentiation of stress BFU-E requires three signals, BMP4, SCF and hypoxia[12]. Control treated mice start to recover earlier than splenectomized mice, which is due to stress erythropoiesis in the spleen. This response may produce enough new erythrocytes to alleviate the tissue hypoxia in the liver, which would significantly decrease the expansion and differentiation of stress BFU-E in the liver. The erythropoietic foci observed in the earlier experiments [23,24] would be caused by increased tissue hypoxia due to the prolonged anemia, which would promote the expansion and differentiation of stress BFU-E. The expansion of stress BFU-E in the livers of control mice is regulated by BMP4 dependent signaling because control *f/f* mice exhibit a clear defect in the expansion of liver stress BFU-E.

Our analysis also showed that the erythropoietic foci are present in areas of BMP4 expression in the livers of splenectomized mice. Previous work demonstrated that erythropoietic foci were localized to the peri-portal regions of the liver acini in PHZ treated mice, however no mechanism was proposed to explain this localization[23,24,34]. This region corresponds to zones 2 and 3, regions of the liver functional unit, which normally have higher O₂ concentrations and do not express BMP4, but in response to acute anemia BMP4 expression is up-regulated in these regions. Our data would suggest that the localization of erythropoiesis to this area is due to tissue hypoxia, which leads to expanded expression of BMP4. Tissue hypoxia also acts in concert with BMP4 and SCF to promote the proliferation and differentiation of stress BFU-E [12].

The spleen maintains a resident population of BMP4 responsive stress progenitors[8]. The livers of untreated splenectomized and control mice contained very few stress BFU-E. Furthermore, treatment of liver mononuclear cells from untreated mice with BMP4 did not increase the number of stress BFU-E (data not shown), which suggests that the liver does not maintain a resident population of BMP4 responsive progenitors. These observations suggest that progenitors migrate into the liver during the recovery period, which may explain the delayed kinetics of recovery in splenectomized mice.

Unlike mice, splenic erythropoiesis is not feature of human hematopoiesis. However in certain pathological conditions, extramedullary erythropoiesis is observed in humans[13–21]. For example in primary myelofibrosis, splenic and hepatic erythropoiesis has been observed. In some cases splenectomy in these patients leads to increased liver erythropoiesis, which can cause liver dysfunction [19]. In general, pathologies that limit medullary hematopoiesis lead to extra-medullary hematopoiesis, but the mechanisms that regulate these responses are not known and the potential role of BMP4 in this process remains to be shown. Although we have characterized the BMP4 dependent stress erythropoiesis pathway in some detail in mice[6,8, 12], this stress response pathway has not been identified in humans. Work from Luck et al. [35] identified a human “stress erythroid “ progenitor that produced high levels of fetal hemoglobin when differentiated in vitro. These progenitors were characterized by the expression CD34, KIT, CD71 and glycophorin A on their surface. This combination of markers is similar to the Kit+CD71+Ter119+ stress progenitors that we identified in the murine spleen [12]. Future work will be needed to determine whether these putative human stress erythroid progenitors respond to BMP4 and whether BMP4 signaling plays a role in the establishment of extramedullary erythropoiesis in patients.

Overall our data suggest that the BMP4 dependent stress erythropoiesis pathway may be a common mechanism for regulating extra-medullary erythropoiesis in adult mice. These data show that the adult spleen and the fetal and adult liver contain a specialized microenvironments that supports the expansion of stress erythroid progenitors at times of acute erythropoietic need.

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Figure 1. Recovery from PHZ induced acute hemolytic anemia

Splenectomized C57BL/6 and C57BL/6 control mice were treated with PHZ to induce anemia. Hematocrit measurements were done at the indicated times. The data shown are the average $\pm$ standard deviation of at least 3 mice per time point. * $p < 0.05$, ** $p < 0.01$.

Figure 2. Analysis of BFU-E in the bone marrow and liver during the recovery from PHZ induced anemia

(A). Analysis of BFU-E in the bone marrow during the recovery from acute anemia. Bone marrow cells were plated in methylcellulose media supplemented with Epo (3U/ml) and IL-3 (50ng/ml). The data are the number of BFU-E per 1×10^5 bone marrow cells. (B) Analysis of BFU-E in the liver during recovery from acute anemia. Liver mononuclear cells (1×10^4 per well) were plated in methylcellulose media supplemented with Epo + IL-3 (left) or Epo alone (right). Statistically significant differences are indicated on the figure. The figure shows a representative experiment of 2 independent experiments. The data shown are the average $\pm$ standard deviation of at least 3 mice per time point.

Figure 3. Analysis of BMP4 expression in the liver during recovery from acute anemia
 (A) Hematoxylin and eosin stained liver sections from C57BL/6-splenectomized and C57BL/6 control mice (Top) and C57BL/6-f/f splenectomized and C57BL/6-f/f control mice (bottom) at indicated time points following a PHZ induced acute anemia. Examples of the darker staining erythropoietic clusters are shown in gray boxed area marked in the day 6 in C57BL/6 splenectomized and control mice and on day 8 in the C57BL/6-f/f splenectomized and control mice. Magnified views of the gray boxed regions are shown to the right of the panels. At least two independent livers were analyzed at each time point. Each liver was fixed, cut into several pieces and sectioned so that each section had fields from multiple areas of the liver. 2–3 sections per time point were analyzed and representative fields shown. (B) Schematic of liver acinus

(functional interpretation of liver organization) formed from classical liver lobules. Blood enters the liver from portal triads composed of portal vein, hepatic artery and bile duct flows towards the central vein. This creates a gradient of oxygen concentration ranging highest in zone 1 (1), intermediate in zone 2 (2), to lowest in zone 3 (3). (C) Liver sections from C57BL/6-splenectomized and C57BL/6 control mice and (D) C57BL/6-*f/f* splenectomized and C57BL/6-*f/f* control mice recovering from an acute anemia stained with anti-BMP4 antibodies and HRP-conjugated secondary antibodies (BMP4) or secondary alone (control). At least two independent livers were analyzed at each time point. Each liver was fixed, cut into several pieces and sectioned so that each section had fields from multiple areas of the liver. 2–3 sections per time point were analyzed and representative fields shown. (*) denotes portal vein of triad composed of portal vein, hepatic artery and bile duct. Arrow points to a central vein. BMP4 staining is brown, with blue hematoxylin counter-stain. Note an example of how BMP4 expression remains expanded into zones 1 and 2 (bracket) into Day 6 post-PHZ in splenectomized mice, while it returns to exclusively zone 3 (3) in wild-type mice by 4 Days post anemia.

Figure 4. Recovery of splenectomized *f/f* mice from acute anemia is compromised

(A). Splenectomized C57BL/6-*f/f* and C57BL/6 control mice were treated with PHZ to induce acute anemia. Hematocrit was measured on the indicated days. *p<0.05, **p<0.01, ***p<0.001. (B) Survival curve of splenectomized C57BL/6-*f/f* and C57BL/6 control mice were treated with PHZ. Recovery from PHZ induced anemia was done in 2 independent experiments. 4–6 mice were used per time point. (C) Analysis of BFU-E in the liver of splenectomized C57BL/6-*f/f* and C57BL/6 control mice were treated with PHZ. Liver mononuclear cells (1×10^4 per well) were plated in methylcellulose media supplemented with Epo + IL-3 (left) or Epo alone (right). Statistically significant differences are indicated on the figure. The figure shows average $\pm$ standard deviation of four mice per time point.