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Accessibility
Macrophages support pathological erythropoiesis in Polycythemia Vera and Beta-Thalassemia

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Abstract

Regulation of erythropoiesis is achieved by integration of distinct signals. Among these, macrophages are emerging as erythropoietin-complementary regulators of erythroid development, particularly under stress conditions. We investigated the contribution of macrophages for physiological and pathological conditions of enhanced erythropoiesis. We utilized mouse models of induced anemia, Polycythemia vera and β-thalassemia in which macrophages were chemically depleted. Our data indicate that macrophages contribute decisively for recovery from induced anemia as well as the pathological progression of Polycythemia vera and β-thalassemia by modulating erythroid proliferation and differentiation. We validated these observations in primary human cultures, showing a critical direct impact of macrophages on proliferation and enucleation of erythroblasts from healthy individuals and Polycythemia vera or β-thalassemic patients. In summary, we identify a new mechanism that we named “Stress Erythropoiesis Macrophage-supporting Activity” (SEMA) that contributes to the pathophysiology of these disorders and will have critical scientific and therapeutic implications in the near future.
Introduction

Erythropoiesis is a dynamic and complex process during which erythroid progenitors develop into enucleated red blood cells (RBCs). This multistep program is tightly regulated to allow for constant replenishment of the highly abundant but short-lived RBCs. At steady state erythropoiesis is mainly orchestrated by erythropoietin (Epo), although several additional growth factors have been implicated in this process. In addition, recent evidences indicate that erythroid development is controlled by the neighboring microenvironment. Erythroid cells have long been described to develop in a specialized niche, the “erythroblastic island”, composed of a central macrophage surrounded by erythroblasts (EBs) at different stages of maturation. Macrophages within these structures are hypothesized to carry out essential physiological functions including iron recycling from senescent RBCs and engulfment of nuclei extruded during erythroblast (EB) enucleation. Moreover, several lines of data suggest that macrophages also promote the proliferation and survival of maturing EBs. These effects seem to be dependent on EB-macrophage contact, suggesting that adhesion molecules or paracrine factors secreted within the erythroid niche could be important effectors of these regulatory events. Consistent with this hypothesis, macrophage or EB derived factors and different adhesion molecules known to be important for erythroblastic island formation have been shown to regulate erythropoiesis, particularly during embryogenesis or under conditions of stress erythropoiesis.

Even though erythropoiesis is an extremely active process, it can be further stimulated under conditions of anemia, a process commonly known as stress erythropoiesis. During stress erythropoiesis, erythroid development extends to extramedullary sites (mainly the spleen and liver), leading to expansion of the erythroid progenitor pool, recruitment of iron and increased reticulocytosis and RBC production. Unlike steady state erythropoiesis, which seems to be relatively insensitive to microenvironmental changes, stress erythropoiesis is highly dependent on integrating signals from both the Epo/EpoR/Jak2/Stat5 axis and those activated within the EI, including BMP4/SMAD5 and integrin signaling. Interestingly, the pathophysiology of several disorders resembles a “chronic” state of stress erythropoiesis. This includes two seemingly dichotomous diseases, Polycythemia vera and β-thalassemia. Polycythemia vera is characterized by extremely elevated erythrocytosis associated with the constitutively active JAK2V617F mutation. In contrast, β-thalassemia is characterized by anemia (due to low β-globin synthesis) and expansion of the pool of erythroid progenitors. Furthermore, mouse models and human patients of both diseases share features characteristic of stress erythropoiesis, including splenomegaly, expansion of the erythroid progenitor pool, and elevated reticulocytosis. These features stem from elevated activation of JAK2 downstream of EPOR (arising from the JAK2V617F mutation in Polycythemia vera or elevated EPO levels in β-thalassemia) and can be limited by the administration of JAK2 inhibitors. Even though these disorders can be classified as “chronic” stress erythropoiesis conditions triggered by genetic lesions, the contribution of macrophages to their pathophysiology has never been investigated.

In this study we explore the role of macrophages during stress and pathological erythropoiesis. Our results indicate an incisive contribution of macrophages under different stress conditions (anemia, EPO administration, Polycythemia vera and β-thalassemia). Interestingly, macrophage-depletion studies in mouse models of Polycythemia vera or β-thalassemia lead to a positive pathological outcome, reversing some key features linked to both diseases. We further extend these observations to human samples and present evidences supporting the need for direct contact between macrophages and erythroid progenitors for these effects, suggesting that macrophage-erythroblasts adhesion interactions within the
erythroblast island are essential for RBC development in physiological and disease conditions.

Results

Clodronate-containing liposomes effectively eliminate splenic red pulp and bone marrow macrophages

In order to study the role of macrophages during erythropoiesis, we depleted macrophages in vivo by administering clodronate-encapsulated liposomes (referred hereafter as “clodronate”). We confirmed that a single intravenous (IV) injection of clodronate eliminated macrophages from the spleen and bone marrow (BM), as seen by a significant decrease in F4/80 expression in these organs (See Materials and methods and Supplementary Fig. 1). Moreover, our data shows that this approach targets various subsets of macrophages, as previously shown, including those co-expressing the macrophage-specific marker F4/80 and the adhesion molecule Vcam1, markers identifying macrophages in the erythroblastic islands. No significant effects could be seen on the percentage of other myeloid lineages in these organs, suggesting that this approach is targeting specifically the macrophage lineage (Supplementary Fig. 1).

Clodronate treatment markedly impairs the response to stress erythropoiesis in WT mice

To understand the role of macrophages during steady state erythropoiesis, WT mice were treated with clodronate every two weeks for a total of 12 weeks. This treatment induced a mild compensated iron-deficiency anemia, characterized by decreased hemoglobin and RBC mean hemoglobin content (MCH), but not by reduced RBC (Supplementary Fig. 2a), reticulocyte counts (not shown), spleen weight or significant changes in erythroid profiles by FACS analysis (Supplementary Fig. 2b and 2c). The reduction of MCH, associated with decreased transferrin saturation (not shown), indicates that chronic depletion of macrophages at steady state reduces iron availability and hemoglobin synthesis, rather than total RBC production. Moreover, clodronate-treated mice presented an elevated serum erythropoietin compared to controls at the point when anemia started to develop (Supplementary Fig. 2d), demonstrating that anemia was not associated with inappropriate erythropoietin signaling.

Considering the limited contribution of macrophages to steady state erythropoiesis, we investigated the potential impact of macrophage depletion under conditions of increased erythropoiesis. Mice treated with clodronate while subjected to phlebotomy-induced anemia (Clod (day 0) group) showed impaired recovery from anemia, with reduced reticulocytosis, compared to phlebotomized control animals (Fig. 1a). This was associated with limited erythropoiesis in the BM and spleen in clodronate-treated mice as compared to controls, as seen by flow cytometry (Fig. 1c and Supplementary fig. 3a and b). A second group of mice were treated with clodronate 4 days after induction of anemia (Clod (day 4) group), when elevated erythropoietic activity and splenomegaly can be observed (Fig. 1b and 1c). Compared to phlebotomized controls, Clod (day 4) mice exhibited a significant reduction in reticulocyte counts, splenomegaly and in the percentage of erythroid precursors in the BM and the spleen as early as day 6 (Fig. 1a–d and Supplementary Fig. 3c and 3d). Clodronate administration in this setting also led to the appearance of a distinguishable CD71highTer119low population in the flow cytometry profiles. This population revealed to be mostly EBs (CD71highTer119lowCD45low) undergoing cell death (Fig. 1e and Supplementary Fig. 4). These effects resulted in an impaired recovery from anemia in Clod (day 4) animals (Fig. 1a), demonstrating that macrophages are important for both expansion and maintenance of elevated erythroid activity in conditions of anemia. Based on these observations, we also investigated whether macrophages are required to support increased...
erythroid activity following administration of human recombinant EPO (EPO). Indeed, clodronate administration impaired erythroid expansion in the spleen as well as RBC and reticulocyte production in response to EPO supplementation (Supplementary Fig. 5). Altogether, these data strongly suggest that, under conditions of stress erythropoiesis, signals from the erythroid niche seem to complement those downstream of the Epo-receptor to fully support erythropoietic activity, independently from the hypoxic status.

**Impairment of recovery from induced anemia in clodronate-treated mice is not associated with impaired iron delivery to erythroblasts**

Macrophages have been shown to play an important role in the maintenance of normal iron homeostasis, as they recycle iron from senescent RBCs. Considering this, we aimed to determine if slow recovery from anemia was associated with decreased iron availability for erythropoiesis. We first analyzed the serum iron parameters and observed that, following phlebotomy, clodronate-treated WT mice presented decreased serum iron and transferrin saturation values compared to PBS controls (Supplementary fig. 6a and 6b), corroborating the notion that macrophages play an important role on iron recycling. We then investigated if the impaired recovery from anemia following macrophage depletion could be exclusively attributed to limited iron availability. Considering that dietary iron and hepcidin have been previously shown to play a crucial role during the recovery phase from phlebotomy-induced anemia\(^\text{27}\), we hypothesized that dietary iron supplementation or mouse models of increased dietary iron uptake (Hfe-KO and Hamp-KO) would be able to compensate for iron deficiency following a single clodronate administration. In fact, clodronate treatment in phlebotomized Hfe-KO, Hamp-KO or WT animals fed an iron-rich diet, did not alter iron supply to erythroid cells, as seen by similarly elevated serum iron levels during all the phases of the recovery in PBS- or clodronate-treated mice of each group (Supplementary fig. 6a and 6b). To a certain extent, elevated serum iron parameters allowed the iron-loaded animals to recover faster than WT mice on regular diet from phlebotomy-induced anemia (Supplementary fig. 6c). However, compared to WT controls, the faster recovery of hemoglobin values in iron-loaded animals was predominantly associated with elevated MCH levels rather than an increase in RBC numbers (Supplementary fig. 6c–e). In addition, erythroid expansion in the bone marrow and spleen was impaired by macrophage depletion in iron-loaded mice following phlebotomy (Supplementary fig. 7), likely accounting for the delay in recovery from anemia compared to iron-loaded or non-iron loaded WT controls (Fig. 1e and Supplementary fig. 6c–d). Altogether these observations indicated that elevated serum iron levels are largely insufficient to compensate for the absence of macrophages in sustaining erythroid proliferation and RBC synthesis during recovery from anemia. These data supports the notion that iron is important to sustain stress erythropoiesis, but also that pathways triggered by macrophages are quantitatively important in erythropoietic response following phlebotomy-induced anemia, independently from the role of macrophages in erythroid-iron recycling.

**Clodronate improves phenotype of mice affected by Polycythemia vera**

Considering the crucial role that macrophages play under conditions of increased erythropoietic activity, we investigated whether macrophages are also involved in the pathophysiology of Polycythemia vera utilizing a transgenic line (Jak2\(^{2V617F/+}\)) carrying a Cre-inducible Jak2\(^{2V617F}\) mutation.\(^{40}\) These animals exhibit a transplantable Polycythemia vera-like phenotype, which is mediated by the constitutive activation of Jak2\(^{40}\). Accordingly, we generated a cohort of Polycythemia vera animals engrafting Jak2\(^{2V617F/+/+}\) VAV-Cre double transgenic BM into normal mice. As early as two weeks post-engraftment, mice transplanted with Jak2\(^{2V617F/+/+}\)VAV-Cre double transgenic bone marrow developed features characteristic of Polycythemia vera, including elevated hematocrit (HCT), increased RBC counts and reticulocytosis. In contrast to PBS-treated mice, weekly clodronate
administration beginning 16 days post-engraftment rapidly normalized the HCT and RBC counts in these animals (Fig. 2a). Furthermore, clodronate-treated mice showed significant evidence of decreased erythropoietic activity as indicated by reduced reticulocytosis, decreased extramedullary hematopoiesis (EMH) in the bone marrow and spleen, splenomegaly (Fig. 2b–e) and the number of Epo-independent BFU-E, as assessed by colony assay (Supplementary Fig. 8). The same reduction in erythrocytosis and EMH was achieved when clodronate administration was initiated 2 months post-BMT, a point at which the Polycythemia vera phenotype is fully established (not shown), indicating that macrophage depletion not only prevents, but also reverses the phenotype in these animals.

**Short-term clodronate treatment ameliorates IE and anemia in β-thalassemic animals (Hbb\(^{th3/^+}\))**

Our results indicate that macrophages are required to support increased erythropoiesis under “transient” (following phlebotomy or EPO administration) or “chronic” (as in Polycythemia vera) stress conditions. Another disease in which erythropoiesis is constitutively enhanced to compensate for the chronic anemia is β-thalassemia. However, erythropoiesis in this disorder is ineffective, characterized by an increased number of erythroid progenitors that fail to generate enough RBCs as a consequence of premature death and reduced differentiation\(^{37–39}\). Therefore, we investigated how macrophages influence IE in a mouse model of β-thalassemia intermedia (Hbb\(^{th3/^+/}\))\(^{40}\), exhibiting anemia and splenomegaly.

Strikingly, 40 hours after a single clodronate injection, Hbb\(^{th3/^+}\) mice showed improvement of their anemia, characterized by increased hemoglobin and RBC as compared to Hbb\(^{th3/^+}\) control mice (Fig. 3a). Moreover, the spleen size was also significantly decreased by 32% in clodronate-treated compared to PBS-treated Hbb\(^{th3/^+}\) animals (Fig. 3b). Some of these effects could be seen as early as 20 hours after clodronate administration (Supplementary Fig. 9). In both cases, these improvements were not associated with increased apoptosis of the erythroid progenitors (Supplementary Fig. 10), but rather with an improvement of IE, as indicated by reduction in the number of erythroid progenitors and proportional increase in differentiated erythroid cells in both the spleen and the BM (Fig. 3c–d and Supplementary Fig. 11 and 12). In agreement, clodronate treatment led to a reduction in the number of cycling EB in the spleen (Supplementary Fig. 13), possibly accounting for decreased splenomegaly. These results indicated that, paradoxically, macrophages impair efficient erythroid development in β-thalassemia. Interestingly, these changes were associated with increased hepcidin expression in the liver along with decreased serum iron levels (Supplementary Fig. 14), which have been shown to improve IE in Hbb\(^{th3/^+}\) mice\(^{50}\). In order to understand if the effect on IE seen after clodronate administration is solely mediated by reduced iron availability\(^{50,51}\), we iron-loaded β-thalassemic animals by administration of high-iron diet or by generating mice that lacked hepcidin expression (Hbb\(^{th3/^+}\)-Hamp-KO mice). Considering that in mice, dietary iron absorption accounts for about 50% of iron in circulation\(^{52,53}\), we hypothesized that dietary iron supplementation could suffice to compensate for decreased iron recycling following clodronate administration. At steady state we observed that serum iron values were elevated in both models compared to Hbb\(^{th3/^+}\) controls. However, unlike the situation in non-iron-loaded Hbb\(^{th3/^+}\) mice, macrophage-depletion in iron-overloaded Hbb\(^{th3/^+}\) animals did not lead to any decrease in serum iron availability (Supplementary Fig. 15a and 16a). However clodronate-administration still resulted in improvements in both erythroid differentiation (Supplementary Fig. 15b–d and 16b–d) and splenomegaly (28% reduction in iron supplemented Hbb\(^{th3/^+}\) mice and 12% reduction in Hbb\(^{th3/^+}\)-Hamp-KO mice), suggesting an iron-independent function of macrophages in stimulating erythropoiesis. Furthermore we also tested if supplementation of Hbb\(^{th3/^+}\) animals with parenteral iron-dextran could compensate for the effect seen after clodronate administration. While this treatment did not increase serum iron values at steady state, it prevented drop of these parameters following clodronate administration in Hbb\(^{th3/^+}\) mice.
animals (Supplementary Fig. 17a). Even in this setting, macrophage-depletion resulted in a reduction of EMH (Supplementary fig. 17b–d) and splenomegaly (30% reduction). While the role of iron in ineffective erythropoiesis in β-thalassemia has been previously shown\textsuperscript{50,51}, our data suggest that macrophages further contribute for the development of EMH and splenomegaly in this disorder, independently from modulation of erythroid iron availability.

**Chronic clodronate treatment improves IE and anemia in β-thalassemia intermedia (Hbb\textsuperscript{th3/+})**

In order to determine how long-term macrophage depletion affects IE, we administered clodronate to Hbb\textsuperscript{th3/+} mice up to 12 weeks. This treatment led to a significant improvement of anemia and RDW (Fig. 4a). Furthermore, despite an increase in the number of circulating RBCs in clodronate-treated mice, reticulocyte counts decreased over time, indicating that erythropoietic activity was reduced (Fig. 4a). This apparent contradiction was resolved when we observed that macrophage depletion increased the RBC lifespan to levels similar to those observed in normal mice (Fig. 4b). However, the increased lifespan of the RBCs in clodronate-treated mice was not associated with deficient erythrophagocytosis, but rather with an improvement in the RBC phenotype as indicated by a series of additional observations: first, the lifespan of RBCs derived from clodronate-treated Hbb\textsuperscript{th3/+} mice was significantly extended compared to that of RBCs derived from PBS-treated animals, after transfusion into GFP-positive WT animals with an intact reticuloendothelial system (Fig. 4c); second, the morphology of RBCs was improved (Fig. 4d); third, the MCH of the RBCs was decreased in clodronate Hbb\textsuperscript{th3/+} treated-mice (Fig. 4a), suggesting that these cells take up less iron, with potential beneficial effects on hemichrome formation\textsuperscript{50,51}. In fact, RBCs derived from clodronate-treated Hbb\textsuperscript{th3/+} mice exhibited reduced accumulation of α-globin chains in the RBC membrane compared to Hbb\textsuperscript{th3/+} PBS liposome-treated controls (Fig. 4e).

Chronic clodronate treatment markedly suppressed splenomegaly, with total spleen size reversing within two months to levels comparable to WT mice (Fig. 4f). The decrease in splenomegaly was erythropoietin-independent (Supplementary fig. 18), being associated with a reduction of EMH and restoration of splenic architecture (Fig. 4g–h and supplementary fig. 19).

**Primary human macrophages stimulate proliferation and delay enucleation of primary human erythroblasts**

Considering the interesting data obtained in mouse models of Polycythemia vera and β-thalassemia, we investigated how primary human macrophages influenced development of human erythroblasts ex-vivo. For that we expanded hematopoietic progenitors from patients affected by Polycythemia vera (JAK2\textsuperscript{V617F} positive), β-thalassemia and healthy individuals and evaluated their proliferative potential when differentiated into the erythroid lineage in the presence of macrophages. Irrespective of their genotype, EB cultured in the presence of macrophages exhibited increased proliferation compared to those cultured alone (Fig. 5a). This effect was associated with an increased percentage of cells cycling as well as a decreased number of apoptotic EB when macrophages where utilized in the co-culture system (Fig. 5b and Supplementary Fig. 20). We then investigated if direct macrophage-erythroblast contact was required for this effect, by performing trans-well experiments. We observed that the positive effect exerted by macrophages on erythroid proliferation was abrogated by 75% in the trans-well co-cultures, suggesting that direct macrophage-erythroblast interaction is required (Fig. 5c), even though some secreted factors might also be involved. We then looked at the effect of the co-culture on erythroid differentiation by assessing the expression of different cell surface markers. First we observed that macrophages did not have a dramatic effect on the morphological appearance of
erythroblasts, and similarly did not significantly affect the expression of the erythroblast developmental markers Glycophorin A (GPA), CD44, CD117 and Band-3 (Supplementary fig. 21). However, when we looked at enucleation and beta1-integrin expression, we observe that at latter time points (day 8), there was a significant reduction in the number of enucleated EB in the co-cultures, as well as retention of higher beta1-integrin surface levels in a greater number of EBs (Fig. 5d and supplementary fig. 22). These observations are consistent with our results in the mouse models, supporting a model where macrophages promote erythroid proliferation and survival, while reducing EB differentiation. Moreover, our data further highlight the critical role of the erythroid microenvironment, particularly the macrophage-EB interaction(s), in the regulation of pathological erythropoiesis, and extends the relevance of our findings in human samples.

Discussion

Macrophages are historically classified as immunological cells whose main function is immune surveillance. However, several lines of evidence suggest additional trophic functions for macrophages in different tissues. One such function is the regulation of erythroid development. However, no one has ever investigated the contribution of macrophages to pathological erythropoiesis and evaluated the therapeutic potential of targeting their function in disease states associated with disordered erythropoiesis.

Our present data underscore the central role that macrophages play under conditions of increased erythropoietic activity, such as induced anemia or EPO administration. We previously showed that iron availability is crucial for the recovery from induced anemia. Our data further expand on this hypothesis, supporting the notion that elevated iron supply can be beneficial in conditions of anemia. Moreover, our results show that, even under conditions where erythroid iron supply is elevated, lack of macrophages delays recovery from induced anemia, indicating that macrophages play an important role in supporting stress erythropoiesis independent from providing iron to EBs. Altogether our data demonstrate that, even though EPO3,55 and iron are rightfully recognized as master regulators of erythropoiesis, they are not sufficient to fully support EB proliferation and survival during stress erythropoiesis. Under such conditions the iron-independent “Stress Erythropoiesis Macrophage-supporting Activity” (SEMA) is also required for proper erythroid activity (Fig. 6).

We further extended the concept of macrophage-dependent erythropoiesis to pathological conditions associated with elevated erythropoietic activity, such as Polycythemia vera or β-thalassemia. Polycythemia vera is a clonal stem cell disorder in which the somatic JAK2V617F mutation leads to a hyperproliferative phenotype. While our data do not question the importance of the JAK2V617F mutation for Polycythemia vera, it provides an additional degree of complexity to this disorder. We clearly demonstrate that, macrophage-depletion in mice carrying the Jak2V617F mutation reversed some of the key features of Polycythemia vera, including splenomegaly, reticulocytosis, erythrocytosis and elevated hematocrit. Furthermore, we show that proliferation of cells derived from JAK2V617F patients is increased when they were co-cultured with macrophages. Thus, we propose a new model for Polycythemia vera progression in which the JAK2V617F mutation functions as primer for the Polycythemia vera phenotype, but a permissive niche and SEMA are required for full manifestation of the erythroid phenotype in vivo. This model represents a clear parallel to the current understanding of tumor biology. Also in this case, an oncogenic mutation is considered to be the primary event leading to tumorigenesis. However, the role of tumor associated macrophages supporting tumor progression and metastatic spread is now uncontested. This is the first description of what we believe to be an important mechanism...
contributing for the pathophysiology of Polycythemia vera that could have significant therapeutic implications for the management of this disorder.

Erythropoiesis in β-thalassemia is characterized by 4 pillars: (1) increased proliferation; (2) ineffective differentiation, which are associated with modulation of the JAK-STAT pathway; (3) reduced survival of EBs; and (4) limited RBC lifespan. All of these features are modulated by macrophages, making the interpretation of our studies more complicated. On the one hand, our data indicated that macrophage depletion directly influences erythroid development in β-thalassemia by decreasing erythroid proliferation and increasing their differentiation, leading to reduced reticulocytosis (as in stress erythropoiesis and Polycythemia vera mice), splenomegaly and EMH (Fig. 6). The effect on erythroid expansion and splenomegaly is seen very rapidly following macrophage depletion, pointing to a direct effect of macrophages in modulating signaling pathways controlling erythroid proliferation and differentiation. In agreement, we show that these effects are seen independently from the effects of clodronate treatment in erythroid iron availability (in Hamp-KO, and iron supplemented thalassemic mice). Moreover, we also demonstrate in human samples, that macrophages directly promote proliferation and limit enucleation of human primary erythroblasts. On the other hand, we show that clodronate treatment impairs iron delivery to maturing erythrocytes, as seen by reduction of serum iron levels following clodronate administration. Similarly to what was previously shown, limited iron availability likely contributes for a reduction in hemichrome formation, and consequently increased RBC lifespan. Thus, despite the fact that production of reticulocytes decreases, the number of RBCs is increased because their survival and lifespan are normalized (Fig. 6). Altogether our data supports a model in which macrophages depletion positively influences erythropoiesis in β-thalassemia by two distinct mechanisms: a) direct modulation of erythroid activity by limiting SEMA; and b) reduced iron availability. We believe that targeting these two pathways would be of greater therapeutic benefit than targeting each of them individually.

In summary, our data support a model where SEMA stimulates signaling pathways that complement Epo/EpoR/Jak2 signaling and iron supply and are essential for erythroid expansion and differentiation (Fig. 6). Without this support the proliferative potential of erythroid cells diminishes substantially. Thus, while loss of SEMA seems to be detrimental during anemia in healthy individuals, it likely decreases the pathological features of disorders associated with enhanced erythroid activity, like Polycythemia vera or β-thalassemia. We believe that these findings are of great interest, as they could pave the road for the identification of novel therapeutic approaches complementary to those currently being employed in these disorders.

Online methods

Animals and procedures

Mice on a c57Bl/6 background were maintained and bred at the Weill Cornell Medical College animal facility. All procedures were performed according to approved protocols.

We induced stress erythropoiesis in 2- to 5-month-old WT animals by phlebotomy (400ul/25g body weight) performed under anesthesia on 3 consecutive days, followed by administration of equal volume of normal saline, as previously described. Recovery from anemia was assessed by withdrawing 50–100 μl of blood every 2–3 days and performing complete blood cell counts (CBCs). An iron-rich diet (supplemented with 2% carbonyl iron) was given to a subset of mice one week prior phlebotomy. These animals were kept on the high-iron diet throughout the recovery. We also stimulated erythropoietic activity WT mice by daily intra-peritoneal (IP) administration of 50 units of hrEPO (Epogen, Amgen, CA) for

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8 days. To determine how macrophages influence the response to these stress conditions, we administered clodronate to phlebotomized or EPO-treated mice. Clodronate suspension was prepared as previously described and administered intra-venous (100 μl) according to the injection schemes summarized in Supplementary Fig. 23. We administered PBS-containing liposomes in control animals for all experiments. Clodronate was provided by Roche Diagnostics, Mannheim, Germany.

We utilized mice carrying a Jak2-V617F conditional knock-in crossed with animals expressing cre-recombinase under the control of the hematopoietic vav regulatory elements (VAV-Cre) to generate animals affected by Polycythemia vera. Jak2V617F/+ VAV-Cre double-transgenic mice developed a Polycythemia vera-like phenotype, which was transplantable, as previously demonstrated. In order to generate large number of animals affected by Polycythemia vera we performed bone marrow transplantation (BMT) into lethally irradiated recipients. In brief, 3–5×10⁶ BM cells from Jak2V617F/+ VAV-Cre mice were transplanted by IV injection into the lateral tail veins of lethally irradiated (9.5Gy) c57Bl/6 WT recipients. In order to evaluate the contribution of macrophages for Polycythemia vera, we allowed the Polycythemia vera phenotype to develop following BMT (about 2 weeks). Sixteen days post bone marrow transplant we started weekly IV administration of 200 μl of clodronate- or PBS-liposomes for a total of four injections (see Supplementary Fig. 23 for details). Progression of hematological parameters was monitored by weekly CBCs.

We utilized Hbbth3/+ animals as a model of β-thalassemia intermedia. The animals used in this study were between 2 to 3 months of age. For some experiments (indicated in the figure legends) Hbbth3/+ animals were generated by BMT similarly to what was previously described. Hbbth3/+ Hamp-KO were generated by transplanting 3–5×10⁶ BM cells from Hbbth3/+ mice into lethally irradiated Hamp-KO animals. Experiments with BMT mice were started 2–3 months post-BMT. Macrophage depletion was performed by IV administration of clodronate, PBS-liposomes being utilized as control. For short-term studies, we analyzed animals 20 and 40 hours after a single clodronate injection (100 μl). Long-term macrophage depletion was performed in Hbbth3/+ and WT mice by IV administration of lower doses of liposomes (50 μl) every two weeks, for up to 12 weeks. We followed hematological parameters by CBC throughout the treatment. For more details on clodronate administration schemes please consult Supplementary Fig. 23. Iron supplementation (dietary or parenteral) was performed as described in supplementary Fig. 15, 17 and 23.

**Hematological studies**

Hematological values were determined as previously described. In brief, we collected blood samples by retro-orbital puncture under anesthesia and CBCs were measured on an Advia 120 Hematology System (Bayer, Tarrytown, NY).

**Flow cytometry analysis of mouse erythroid cells**

We harvested BM and spleen cells as previously described. For erythroid analysis, we incubated single cell suspensions with Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD71, Phycoerythrin (PE)-conjugated anti-mouse CD44 and Allophycocyanin (APC)-conjugated anti-mouse Ter119 antibodies (BD Pharmingen, San Diego, CA) for 15 minutes on ice. Samples were washed with PBS supplemented with 1% BSA and acquired in a FACSCalibur (BD) instrument equipped with a dual-laser. For determination of DNA content, we first stained the cells with the cell-surface markers, washed and then re-suspended in 50μl of diluted (1:5,000) DRAQ5 (Cell Signaling Technology) 10–15 minutes before running. For apoptosis analysis we stained single cell suspensions with PE labeled anti-mouse CD71, APC-conjugated anti-mouse CD44 and Pacific Blue-conjugated...
anti-mouse Ter119 antibodies (BD Pharmingen, San Diego, CA) for 15 minutes on ice. After washing, cells were incubated with 7AAD and FITC-labeled Annexin V (BD Pharmingen) in 100μl of 1x binding buffer according to the manufacturer’s instruction. For cell cycle analysis, we utilized the APC-BrdU flow kit (BD Pharmingen), according to the manufacturer’s instructions. In brief, 1 mg of BrdU was administered to mice by IP injection and BM and spleen were harvested 1 hour post BrdU administration. Single cell suspensions were first incubated with FITC-labeled anti-mouse CD71, PE-labeled anti-mouse CD44 and Pacific Blue-conjugated anti-mouse Ter119 antibodies (BD Pharmingen, San Diego, CA) as described above. Following cell surface stain, we stained cells with 7AAD and APC-conjugated anti-BrdU antibody as described in the kit manual. Samples were run in a FACS Canto II system (BD) equipped with 3 lasers. Analysis was performed using flow-jo software (Tree Star Inc., Ashland, OR).

**Macrophage and myeloid analysis by flow cytometry**

BM and spleen cells were harvested as previously described57. Single cell suspensions were incubated with PE-conjugated F4/80 anti-mouse antibody (eBiosciences, San Diego, CA) and FITC-labeled anti-mouse CD11b or Gr1 antibody (BD Pharmingen, San Diego, CA) in 30% mouse serum in PBS for 30 minutes on ice. For Vcam1 analysis, cells were first stained with purified anti-mouse Vcam1 antibody (BD Pharmigen, San Diego, CA) in PBS, 1% BSA for 30 minutes on ice. After washing, cells were stained with FITC Goat Anti-Rat Ig (BD Pharmigen, San Diego, CA) for 30 minutes on ice and then washed once with PBS, 1% BSA. F4/80 staining was performed with PE-conjugated F4/80 anti-mouse antibody as described above. Samples were washed with 1% BSA in PBS and acquired in a FACSCalibur (BD) instrument equipped with a dual-laser. Analysis was performed using flow-jo software (Tree Star, Inc., Ashland, OR).

**Measurement of RBC lifespan**

RBC lifespan was determined by labeling RBCs with sulfo-NHS-biotin reagent (Pierce, Rockford, IL) as previously described27,50. In brief, we injected mice with 1 mg of sulfo-NHS-biotin intravenously. Aliquots of blood were stained with fluochrome-conjugated streptavidin antibody and analyzed by flow cytometry at different time points for up to 2 weeks post sulfo-NHS biotin administration.

**Measurement of Hbb\textsuperscript{th3/+} RBC survival in WT mice**

To evaluate whether the lifespan of RBCs from clodronate-treated mice \textit{Hbb}\textsuperscript{th3/+} mice was extended compared to that of PBS-treated \textit{Hbb}\textsuperscript{th3/+} mice in mice with an intact erythropagocytic system, we evaluated the survival of RBCs from these groups after transfusion into GFP positive mice, as previously described50. For that we injected 400 μl of blood from each \textit{Hbb}\textsuperscript{th3/+} mouse into previously phlebotomized (about 400 μl) GFP positive mice. Clearance of GFP-negative \textit{Hbb}\textsuperscript{th3/+} RBCs from the peripheral blood of transfused mice was measured by FACS every 6 days for up to 18 days.

**Analyses of \(\alpha\)– and \(\beta\)– globin chains in plasma and on RBC membranes**

To visualize soluble as well as membrane-bound globins, we utilized triton acetic acid urea (TAU) gel electrophoresis as previously described50,58,59. The fraction of sample loaded on the TAU gel was adjusted considering CBC readings, so that the same number of RBCs (150×10\(^6\)) was loaded in each lane.

**Expansion of human CD34positive hematopoietic progenitors**

Peripheral blood mononuclear cells (PBMCs) were separated by ficoll gradient and, after extensive washing with PBS, were resuspended in 600μl of 1% BSA in PBS. We then
added 100 μl of anti-human CD34 beads for 15 minutes at 4°C, after which we passed the cells through an LS magnetic column (Miltenyi Biotech) according to manufacturer’s instructions. Eluted CD34 negative cells were processed for macrophage differentiation (see below). CD34 positive cells trapped in the column were collected by removing the LS column from the magnetic field and eluting with 5 ml of beading buffer. We expanded CD34 positive hematopoietic progenitors in stem-span supplemented with 1% of CC100 cytokine cocktail (Stem Cell technologies), 2U/ml of hrEPO 100 μg ml⁻¹ of streptomycin, 100 U ml⁻¹ penicillin and 10⁻⁶ M dexamethasone. CD34 positive cells were expanded for up to three weeks at low confluency (0.5–1×10⁶ ml⁻¹) to prevent their differentiation.

**Differentiation of human macrophages**

CD34 negative PBMCs were resuspended in RPMI complete (RPMI, 10% FBS, 100 μg mL⁻¹ of streptomycin, 100 U ml⁻¹ penicillin) supplemented with 10 ng ml⁻¹ of human recombinant macrophage colony stimulation factor (hrM-CSF, Peprotech, Rocky Hill, NJ) at 3×10⁶ cells ml⁻¹. We plated three ml of PBMC suspension in each well from a 6-well plates and monocytes were allowed to adhere to the plastic overnight at 37°C, 5% CO₂. The following day we carefully removed the supernatant and replaced it with 3ml of macrophage differentiation medium (RPMI complete supplemented with 25 ng ml⁻¹ of hrM-CSF). Medium was changed every three days and macrophages were differentiated for 7–10 days.

**Erythroid differentiation of human CD34 positive hematopoietic progenitors**

For erythroid differentiation, human CD34 positive cells being grown for up to three weeks as described above, were washed with PBS and resuspended in human erythroid differentiation medium (alpha-Minimum Essential Medium, 30% Hyclone FBS, 100 μg ml⁻¹ of streptomycin, 100 U ml⁻¹ penicillin, 14μM 2-mercaptoethanol, 10 U ml⁻¹ of hrEPO and 1 ng ml⁻¹ of SCF) at 10⁶ cells ml⁻¹. Erythroid differentiation was carried out for 8 days in 6-well plates in the presence of absence of differentiated macrophages. During differentiation, we kept the cells at 2 to 4×10⁶ cells ml⁻¹ by cell counting and refreshed the medium at day 4, 6 and 8. Erythroid differentiation markers, cell cycle, apoptosis and enucleation analysis were performed at these time points by flow cytometry (see below).

**Human erythroid analyses by flow cytometry**

Differentiating human erythroid cells were stained with the differentiating markers PE-labeled CD117, FITC-labeled Glycophorin A (GPA), APC-conjugated CD44 (BD) and PE-Cy5.5-conjugated Band3 for 15 minutes on ice. Cell cycle analysis was performed with the APC-BrdU flow kit (BD) according to the provided protocol. Apoptosis stain with AnnexinV and 7AAD was performed as described in the main text. All samples were analyzed in a FACSCalibur (BD) instrument equipped with a dual-laser.

**Serum iron content**

Serum iron and transferrin saturation were determined using the Iron/UIBC kit from Thermo Electron (Melbourne, Australia) as previously described⁵⁷.

**Serum EPO levels**

Serum EPO levels were determined by ELISA utilizing the kit from R&D Biosystems (Minneapolis, MN) according to the instructions provided.

**Immunohistochemistry analysis**

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Longitudinal sections (4 um) were stained with hematoxylin and eosin (H&E) as previously described⁵⁷. Immunohistochemistry was performed on splenic sections from clodronate- and PBS-
treated Hbbth3/+/ mice using a F4/80 purified antibody on the BONDmaXAutomated Immunostainer (Vision BioSystems, Melbourne, Australia).

**Quantitative real-time PCR**

We extracted RNA from liver samples utilizing the trizol reagent (Sigma, St. Louis, MO) according to the instructions provided. We then quantified RNA samples and utilized 3 ug of total RNA for retrotranscription using the SuperScript III kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Q-PCR for mouse hepcidin and the internal control, GAPDH, were performed as previously described.

**Statistical analysis**

Unless otherwise indicated, statistical differences were calculated with Student’s t test (Microsoft Excel).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. WT mice depleted of macrophages have impaired recovery from induced-anemia
(a) Variation of hemoglobin and reticulocyte values in peripheral blood of phlebotomized WT mice, treated with PBS- or clodronate-liposomes. Clod (day 0) and clod (day 4) represent animals in which clodronate administration was started as day 0 or day 4 of treatment, respectively. (b) Variation in splenomegaly in PBS- or clodronate-treated phlebotomized WT mice. (c) Representative examples of CD71/Ter119 profiles from the BM and spleen of non-phlebotomized WT (left panel), and phlebotomized PBS- (middle panel) or clod (day 0) animals (right panel) at day 4 of treatment. Immature and mature erythroid cells are identified respectively as CD71\textsuperscript{high}Ter119\textsuperscript{high} and CD71\textsuperscript{Low}Ter119\textsuperscript{high} cells. (d) Representative example of CD71/Ter119 profiles from the BM and spleen of phlebotomized PBS- or clod (day 4) animals, analyzed at day 6 of experiment. (e) Analysis of apoptosis in the gate R1 (CD71\textsuperscript{high}Ter119\textsuperscript{low} cells) defined in (d). For a more detailed analysis refer to Supplementary Fig. 3. (f) Variation of hemoglobin levels following phlebotomy in PBS- or clodronate-treated WT mice maintained on an iron-rich diet. In (a) and (f) the results are presented as means ± SEMs of 4–18 age- and sex-matched mice for each time point. Statistical significance is shown by * P ≤0.05, ** P ≤0.01, *** P ≤0.001.
Figure 2. Clodronate treatment reverses phenotype in a mouse model of Polycythemia Vera

(a) Variation of hematocrit, RBC and reticulocyte values in peripheral blood of mice affected by Polycythemia vera (PV) treated with PBS- or clodronate-liposomes. The results are presented as means ± SEMs of 6 PBS- and 8 Clodronate-treated BMT mice for each time point (** P ≤ 0.01, *** P ≤ 0.001 relative to PBS-controls). (b) Variation in splenomegaly in PBS- or clodronate-treated PV mice. The graphic on top shows the average spleen weight (± SEMs) for each group. (c) Upper panel shows a representative example of CD71/Ter119 profiles from the bone marrow and spleen of PBS- or Clodronate-treated PV animals, as described in Fig. 1. In these graphs an erythroid gate was established (R1) and
events in R1 were plotted as CD44/Forward scatter (FSC) profiles \(^5\) (lower panel). Five progressively more mature erythroid populations (I through V in lower left panel) were defined. (d) Quantification of the data shown in (c). Graph on the left shows the percentage of CD71\(^{\text{high}}\)Ter119\(^{\text{high}}\) (C+T+) or CD71\(^{\text{low}}\)Ter119\(^{\text{high}}\) (C−T+) erythroid cells. On the right is shown the percentage of cells in the populations I–V defined in the CD44/FSC plots. (e) Total number of erythroid cells in the erythroid populations defined in (d). In (d) and (e) the data is shown as means ± SEMs of 3 PBS- and 4 clodronate-treated animals. Statistical significance is represented by * \(P \leq 0.05\), ** \(P \leq 0.01\) or *** \(P \leq 0.001\).
Figure 3. Improvement of anemia and IE in Hbb\(^{th3/+}\) animals 40 hours after single clodronate administration
(a) Hematological parameters 40 hours after a single IV administration of clodronate or PBS liposomes. Hemoglobin, RBC counts and mean corpuscular hemoglobin (MCH) are presented as means ± SEMs of 14 PBS- and 10 clodronate-treated mice. (b) Average (± SEMs) spleen weight and illustrative spleen picture harvested from PBS- or clodronate-treated Hbb\(^{th3/+}\) animals. (c) Representative example of CD71/Ter119 (upper panels) and erythroid (R1 gate) CD44/FSC profiles (lower panels) from the BM and spleen of PBS- or clodronate-treated Hbb\(^{th3/+}\) (th3/+ ) animals, as well as strain matched WT mice, similarly to what is described in Fig. 2. (d) Represents the quantification of the data showed in (c). The two graphs on the right show the percentage of immature CD71\(^{high}\)Ter119\(^{high}\) (C+T+) or...
mature CD71^{low}Ter119^{high} (C–T+) erythroid cells in the CD71/Ter119. The two graphs on the left show the percentage of cells in populations I–V defined in the CD44/FSC profiles. The values are presented as means ± SEMs of 9–10 animals per group. Statistical significance is represented by * \( P \leq 0.05 \), ** \( P \leq 0.01 \), *** \( P \leq 0.001 \).
Figure 4. Chronic clodronate administration improves anemia, IE and RBC phenotype in Hbb<sup>th3/+</sup> mice

(a) Variation in hematological parameters during three months of clodronate treatment. Hemoglobin (g dl<sup>−1</sup>), RBC (number of cells x 10<sup>6</sup> μl<sup>−1</sup>), reticulocyte counts (number of cells x 10<sup>3</sup> μl<sup>−1</sup>), MCH (pg) and red cell distribution width (RDW shown in %) are presented as means ± SEMs of 4–14 PBS- and 4–20 clodronate-treated Hbb<sup>th3/+</sup> mice for each time point. (b) RBC lifespan in PBS- or clodronate-treated WT or Hbb<sup>th3/+</sup> (th3/+) mice. (c) Survival of RBC obtained from PBS- (th3/+ PBS) or clodronate-treated (th3/+ clod) Hbb<sup>th3/+</sup> mice when transfused into GFP positive WT mice. Values in (b) and (c) are presented as the average ± SEMs of at least 5 animals for each group. In (a)–(c) statistical significance is defined by * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. (d) Blood smears stained with May-Grunwald-Giemsa solution, showing RBCs morphology of representative WT, PBS-treated (th3/+ PBS) and clodronate-treated Hbb<sup>th3/+</sup> (th3/+ clod) mice at 2 months of liposome treatment. (e) Quantification of RBC membrane-bound globins analyzed by triton acetic acid urea (TAU) gel electrophoresis. The membrane fractions were prepared in parallel from the same number of RBC for each group before loading. PBS- and clodronate-treated Hbb<sup>th3/+</sup> mice (th3/+) were analyzed at two months of treatment. Non-fractionated RBC lysate was loaded in the control lane (STND). (f) Average weight of spleens harvested from WT, and PBS- or clodronate-treated Hbb<sup>th3/+</sup> (th3/+)-treated animals at different time-points. (g) Representative example of the CD71/Ter119 profiles from the BM and spleen of PBS- or clodronate-treated β-thalassemic animals sacrificed at two months of treatment. (h) H&E staining of splenic sections of WT, and PBS- or clodronate-treated Hbb<sup>th3/+</sup> mice. Yellow arrows show white pulp germinal centers. Results in (g) and (h) were reproduced for at least three independent mice per group.
Figure 5. Human macrophages promote proliferation and limit differentiation of primary human erythroblasts

(a) Proliferation of human erythroid precursors when differentiated alone (EB) or in cocultures with human macrophages (EB+Mac). Graphs show a typical example of proliferation of cells from a healthy donor, a JAK2V617F polycythemia vera patient and a β-thalassemic patient. Each condition was run in triplicate, the values representing the mean ± standard deviation. (b) Cell cycle analysis of human erythroid cells at day 6 of differentiation, when cultured alone (EB) or in the presence of macrophages (EB+Mac). BrdU incorporation was used to determine the percentage of cells in S phase and 7AAD linear analysis enabled quantification of DNA content. The different stages of cells cycle (G0/G1, S and G2/M) are identified in the figures. (c) Proliferation of erythroblasts when
cultured alone (EB), in co-culture with macrophages (EB+Mac) or in co-culture with macrophages in a trans-well format (EB+Mac (TW)). Each condition was run in triplicate and the values are presented as the mean ± standard deviation (*** P ≤ 0.001). In (a) and (c) statistical significance was calculated by two-way ANOVA. (d) Erythroblast enucleation when cultured alone (EB) or in the presence of macrophages (EB+Mac) was assessed at day 8 by DNA staining using DRAQ5.
Figure 6. Model of macrophage function in normal and pathological erythropoiesis

(a) During steady state erythropoiesis macrophages mildly support erythroid development but play a crucial function in maintenance of serum iron availability by controlling hepcidin expression and iron recycling from senescent red cells. Upon macrophage depletion (right panel), erythropoiesis is only slightly reduced, not significantly decreasing RBC production. However, iron metabolism is impaired, leading to low iron availability, reduced hemoglobin synthesis and low MCH. Anemia arises as a consequence of low MCH. (b) Model that describes the (iron-independent) Stress Erythropoiesis Macrophage-supporting Activity (SEMA) under conditions of elevated erythropoiesis associated with anemia, Epo administration or Polycythemia vera due to the Jak2V617F mutation. Under these conditions, SEMA supports erythropoiesis, functioning as a complement to EpoR/Jak2/Stat activation. This allows for a high reticulocytosis and RBC production (left panel). If SEMA is abrogated, erythroid expansion is markedly impaired leading to low reticulocytosis (right panel). Moreover iron availability is also decreased further contributing for impaired erythroid response. While the function of macrophages might be crucial during recovery from anemia, it seems to play a decisive role for the pathophysiology of Polycythemia vera, contributing for high erythrocytosis. (c) Contribution of macrophages to the pathophysiology of β-thalassemia. In β-thalassemia, erythropoiesis is characterized by increased erythroid proliferation and decreased differentiation, which is SEMA dependent (left panel). Absence of macrophages leads to improvement of erythropoiesis by decreasing the proliferation and improving the differentiation of erythroid progenitors (right panel). As macrophage depletion is protracted overtime by repeated administration of clodronate, erythroid iron availability decreases, further contributing for the phenotypic improvement of β-thalassemic RBCs. (d) Model representing how macrophages support stress erythropoiesis.
in the erythroblastic island. EBs adhere to macrophages via different adhesion molecules and these adhesive interactions, along with additional macrophage-secreted factors, activate downstream signaling promoting EB survival, proliferation and controlling differentiation (SEMA activity). In addition, complete support of stress erythropoiesis and hemoglobin synthesis requires Epo and iron, the last one provided by the macrophages by recycling iron from senescent RBCs. Peripheral blood (PB), bone marrow (BM), Spleen (Spl), Liver (Liv), serum iron (SI) Hepcidin (Hpc), erythroid factor (EF) and iron recycling (Fe Rec) are shown as abbreviations in the panels a–c. In a–c erythroid maturation is shown by the connecting lines between each erythropoietic stage, up to RBC in the peripheral blood. Dashed lines represent failure in erythroid differentiation. Arrow stroke represents intensity of each effect (dashed arrows being the lowest).