Macrophages support pathological erythropoiesis in Polycythemia Vera and Beta-Thalassemia


Supplementary figure 1. Elimination of macrophages in the bone marrow and spleen by a single injection of clodronate-liposomes.
Supplementary figure 1. Elimination of macrophages in the bone marrow and spleen by a single injection of clodronate-liposomes.  

(a) Representative examples of myeloid/macrophage lineage analysis by flow cytometry of spleen and bone marrow (BM) cells from PBS- or clodronate-treated animals. Analysis shows Vcam1-F4/80 and CD11b-F4/80 profiles in BM and spleen 40 hours after a single IV injection of liposomes (100μl).  

(b) Representative analysis by flow cytometry of BM myeloid/macrophage lineages utilizing Gr1, CD115, F4/80 and Vcam1, similarly to what was described by Chow et al, J Exp Med, 2011. A first gate was established on the Gr1 negative population (red gate on the first panel from the left) and cells were plotted as F4/80-CD115 (second panel from the left). A clear reduction in the F4/80\textsuperscript{high}CD115\textsuperscript{low} population following clodronate administration (orange gate) was observed. Moreover, when this population was plotted as FSC-SCD, we observed that the SSC\textsuperscript{low} population (representing BM macrophages) is the one most affected by clodronate administration (pink gate on the third panel from the left). Last panel from the left shows the reduction of the F4/80\textsuperscript{high}Vcam1\textsuperscript{high} population, after gating on Gr1\textsuperscript{low} events (red gate).  

(c) Immunohistochemistry of F4/80 (red stain) in spleens of th3/+ mice 40h after a single IV administration of either PBS- or clodronate-liposomes.
Supplementary figure 2. Steady state erythropoiesis in WT mice is not drastically impaired following chronic clodronate treatment. (a) Variation of hematological parameters in wt animals during three months of clodronate administration. Hemoglobin, RBC and MCH are presented as means ± SEMs of 7–13 PBS- and 4–20 clodronate-treated mice for each time point. (b) Average weight of spleens harvested from PBS- or clodronate-treated WT animals after three months of treatment. (c) Representative CD71-Ter119 profiles of BMs and spleens of PBS- and clodronate-treated animals sacrificed after three months of treatment. (d) Serum erythropoietin levels in PBS- and clodronate-treated WT mice 4 weeks after the start of liposome administration. The values are presented as means ± SEMs of 4–5 animals per group. In (a) and (d) statistical significance is shown by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ relative to PBS-controls.
Supplementary figure 3. Clodronate treatment impairs erythroid expansion in BM and spleen of WT mice following phlebotomy. (a) Percentage of immature CD71^{high}Ter119^{high} (C+T+) or mature CD71^{low}Ter119^{high} (C-T+) erythroid cells in the bone marrow and spleen of PBS- or clodronate-treated WT mice 4 days after phlebotomy. Data shows the quantification of erythroid profiles shown in Fig 1c and is presented as means ± SEMs of 4 animals per group. (b) Total number of cells of each erythroid population shown in (a). (c) Percentage of immature CD71^{high}Ter119^{high} (C+T+) or mature CD71^{low}Ter119^{high} (C-T+) erythroid cells in the bone marrow and spleen of phlebotomized WT mice 6 days after phlebotomy. Animals were treated with PBS- or Clodronate liposomes at day 4. Data shows the quantification of erythroid profiles shown in Fig 1d and is presented as means ± SEMs of 6 animals per group. (d) Total number of cells of each erythroid population shown in (c). In all the graphs statistical significance is represented by * P ≤ 0.05, ** P ≤ 0.01 or *** P ≤ 0.001.
Supplementary figure 4. Apoptosis analysis in CD71\textsuperscript{high}Ter119\textsuperscript{low} population in phlebotomized mice. Animals were phlebotomized for three days and liposomes were administered 2 days after the last blood withdrawal (day 4). Analysis was performed 2 days after liposome administration. (a) CD71-Ter119 profiles in PBS- and clodronate-treated mice (Clod (day 4)). A gate was established on the new CD71\textsuperscript{high}Ter119\textsuperscript{low} population, present almost exclusively in Clod (day 4) mice. (b) CD45 analysis of CD71\textsuperscript{high}Ter119\textsuperscript{low} cells from (a) shows that 70-80% of these cells are erythroid (CD45\textsuperscript{low}). (c) Apoptosis analysis of erythroid cells from (b), (red gate), showing an elevated level of apoptosis in this population.
Supplementary figure 5. Macrophage depletion impairs erythropoietic response to hrEPO administration. (a) Variation of hematological parameters after hrEPO (EPO) administration in PBS- or clodronate treated WT mice. Hemoglobin, RBCs, Hematocrit and reticulocyte values are shown as the average ± SEMs of 4 mice per group. (b) Variation in spleen weight in the above mentioned groups. Graphics represent the average ± SEMs of 4 PBS- or clodronate-treated mice for each time point. In (a) and (b) statistical significance is shown by * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 relative to PBS-controls. (c) Representative examples of CD71-Ter119 profiles of BMs and spleens of WT control animals (left panel), mice treated with EPO in combination with PBS-liposomes (middle panel) or EPO in combination with clodronate liposomes (right panel). Analysis was performed 8 days following start of EPO administration.
**Supplementary figure 6.** Clodronate treatment impairs recovery from phlebotomy-induced anemia in iron-supplemented WT, Hfe-KO or Hamp-KO mice.
Supplementary Figure 6. Clodronate treatment impairs recovery from phlebotomy-induced anemia in iron-supplemented WT, Hfe-KO or Hamp-KO mice. In (a) and (b) are shown, respectively, serum iron concentration and transferrin saturation levels of WT controls (WT), WT mice fed an iron-rich diet throughout the recovery (WT + Iron), Hamp-KO and Hfe-KO mice at day 0 and day 7 following the phlebotomy. PBS- and clodronate-treated mice are shown for each group at those time points. (c), (d) and (e) show, respectively, the recovery of hemoglobin, RBC and MCH levels following phlebotomy in the above mentioned groups. The values are presented as means ± SEMs of 4-6 animals per group. In (c) and (d) statistical significance is represented by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ relative to PBS-controls.
Supplementary figure 7. Clodronate administration impairs erythroid expansion following phlebotomy in WT mice independently from iron.
Supplementary figure 7. Clodronate administration impairs erythroid expansion following phlebotomy in WT mice independently from iron. **(a)** Representative CD71-Ter119 profiles of bone marrow and spleens of phlebotomized WT mice at day 4 or day 6 of phlebotomy protocol. WT controls, WT mice treated with clodronate-liposomes, dietary iron-loaded WT mice treated with PBS-liposomes, or dietary iron-loaded WT mice treated with clodronate-liposomes are shown from left to right, respectively. **(b)** Total number of immature CD71^{high}Ter119^{high} (C+T+) or mature CD71^{low}Ter119^{high} (C-T+) erythroid cells in the bone marrow and spleen of the above groups at day 4 or day 6 of phlebotomy regiment. Numbers were calculated based on the profiles shown in **(a)**. Values are presented as means ± SEMs of 3 animals per group. In all the graphs statistical significance is represented by * $P \leq 0.05$, ** $P \leq 0.01$ or *** $P \leq 0.001$. 

Nature Medicine doi:10.1038/nm.3126
Supplementary figure 8. BFU-E colony assay from BM of PV mice treated with PBS- or clodronate-liposomes. Graph shows number of BFU-E colonies formed for each 20,000 BM cells plated in colony-assay medium at different concentration of erythropoietin (Epo). Both BFU-E colonies from PBS- and clodronate-treated PV mice were Epo-independent. However, the total number of BFU-E in JAK2V617F mice treated with clodronate was reduced compared to untreated mice (*** P \leq 0.001 relative to PBS-controls).
Supplementary figure 9. Erythropoietic alterations in \textit{Hbb}^{th3/+} animals 24 hours following a single clodronate administration. (a) Variation of hematological parameters 24 hours after a single liposome administration. Hemoglobin, RBCs, MCH, reticulocyte number and CHr values are shown as average ± SEMs of 5 PBS- and 6 clodronate-treated mice. (b) Representative example of CD71-Ter119 (upper panels) and erythroid (R1 gate) CD44-FSC (lower panels) profiles of BMs and spleens of PBS- or clodronate-treated \textit{Hbb}^{th3/+} (th3/+) animals. Erythroid profiles were analyzed similarly to figure 2 in the main text.
Supplementary figure 10. Clodronate treatment does not induce significant erythroid apoptosis in $Hbb^{th3/+}$ mice.
Supplementary figure 10. Clodronate treatment does not induce significant erythroid apoptosis in Hbb<sup>th3+/+ (th3/+) mice</sup>. (a) Representative example of CD71-Ter119 (upper panels) profiles of BMs and spleens of PBS- and clodronate-treated animals 40 hours after a single liposome administration. Cells within the erythroid gate on the upper panels were further separated into different stages of maturation utilizing the CD44-FSC gating shown in (b). (c) Representative example of apoptosis analysis utilizing Annexin V and 7AAD markers in the different populations defined in (b). No significant changes in the percentage of apoptotic cells could be detected.
Supplementary figure 11. Clodronate administration reduces the total number of erythroid progenitors in the BM and spleen of Hbb\textsuperscript{th3/+} (th3/+) mice. The percentage of cells in each erythroid population from Fig. 3c were utilized to calculate the total number of cells in the spleen (based on spleen weight) and the BM (one femur). (a) Total number of immature CD7\textsuperscript{1high}Ter119\textsuperscript{high} (C+T+) or mature CD7\textsuperscript{1low}Ter119\textsuperscript{high} (C-T+) erythroid cells in the BM and spleen 40h following a single liposome administration. (b) Total number of cells in each stage of erythroid differentiation (I to V) based on FSC-CD44 profiles shown in Fig. 3c. The values are presented as means ± SEMs of 6-7 animals per group. Statistical significance is shown by * \( P \leq 0.05 \), ** \( P \leq 0.01 \), *** \( P \leq 0.001 \).
Supplementary figure 12. Clodronate treatment increases the proportion of enucleated erythroid cells in the BM and spleen of Hbb<sup><i>th3/+</i></sup> (<i>th3/+</i>) mice. (a) CD71-Ter119 profiles in BM and spleen of th3/+ mice 40 hours post administration of PBS- or clodronate-liposomes. The red and blue gates indicate, respectively, total erythroid cells or CD71<sup>low</sup>-Ter119<sup>high</sup> mature erythroid cells. (b) Identification of enucleated (bottom population, indicated by the gate) of total erythroid cells (Red gate in (a)). Discrimination between nucleated and enucleated erythroid cells was achieved using DRAQ5 stain. (c) DRAQ5 analysis of CD71<sup>low</sup>-Ter119<sup>high</sup> cells (blue gate in a). The percentage of enucleated erythroid cells is clearly increased in <i>th3/+</i> mice following clodronate administration in these two populations.
Supplementary figure 13. Cell cycle analysis of spleen and BM cells of *Hbb*th3/+ (th3/+) 40 hours after a single clodronate injection. (a) Flow cytometry showing CD71-Ter119 profiles. (b) Cell cycle analysis on erythroid gate established in (a). BrdU and 7AAD were used to identify the distinct phases of cell cycle as shown (G0/G1, S, G2/M). (c) Quantification of data shown in (b). Data are shown as the means ± SEM of 3 PBS and 4 Clod-treated mice. Statistical significance is shown by * P < 0.05, ** P < 0.01.
Supplementary figure 14. Iron parameters of \( Hbb^{th3/+} \) \((th3/+))\) mice following clodronate treatment. (a) Hepatic \( Hamp \) mRNA expression relative to GAPDH, 40 hours after a single liposome administration. Values are shown as the mean ± SEMs of 4 PBS- and 6 clodronate-treated mice. (b) Serum iron and transferrin saturation 40 hours after a single liposome treatment. Values represent the means ± SEMs of 9 PBS- and 5 clodronate-treated mice.
Supplementary figure 15. Erythropoietic and iron values in iron-supplemented (Dietary) Hbb\textsuperscript{th3/+} (\textit{th3}+/+) animals following clodronate administration. (\textit{a}) Serum iron parameters in dietary iron supplemented \textit{th3}+/+ animals following PBS- or clodronate- liposome treatment. (\textit{b}) CD71-Ter119 (upper panels) and erythroid (R1 gate) CD44-FSC (lower panels) profiles from the spleen of PBS- or clodronate-treated iron-supplemented \textit{th3}+/+ animals. (c) Total number of immature CD71\textsuperscript{high}Ter119\textsuperscript{high} (C+T+) or mature CD71\textsuperscript{low}Ter119\textsuperscript{high} (C-T+) erythroid cells in the spleens of iron supplemented \textit{th3}+/+ mice treated with PBS- or clodronate-liposomes (from top panels in (b)). (d) Total number of cells in each stage of erythroid differentiation (I to V) based on FSC-CD44 profiles shown in (b). In this experiment Hbb\textsuperscript{th3/+} mice were kept on an iron rich diet (supplemented with 2% carbonyl iron), were injected with clodronate liposomes at day 0 (100 µl) and day 5 (80 µl) and were analyzed at day 7.
**Supplementary figure 16.** Erythropoietic and iron values in $Hbb^{th3/+}$ ($th3/+)$ Hamp-KO animals 40 hours following a single clodronate administration.
Supplementary figure 16. Erythropoietic and iron values in $Hbb^{th3+}$ ($th3/+$) $Hamp$-KO animals 40 hours following a single clodronate administration. (a) Serum iron parameters in PBS- or clodronate-treated $th3/+$ $Hamp$-KO animals 40 hours following a single liposome administration. Results show the means ± SEMs of 2 PBS- and 2 clodronate-treated mice. (b) Representative example of CD71-Ter119 (upper panels) and erythroid (R1 gate) CD44-FSC (lower panels) profiles from the BM and spleen of PBS- or clodronate-treated $th3/+$ $Hamp$-KO animals. (c) Total number of immature CD71$^{high}$Ter119$^{high}$ (C+T+) or mature CD71$^{low}$Ter119$^{high}$ (C-T+) erythroid cells in the spleen and bone marrow of $th3/+$ $Hamp$-KO mice treated with PBS- or clodronate-liposomes (from top panels in (b)). (d) Total number of cells in each stage of erythroid differentiation (I to V) based on FSC-CD44 profiles shown in (b). The values are presented as means ± SEMs of 3 animals per group. Statistical significance is shown by * $P \leq 0.05$, ** $P \leq 0.01$. 

Nature Medicine doi:10.1038/nm.3126
Supplementary figure 17. Erythropoietic and iron values in iron-supplemented (parenteral) Hbbth3/+ (th3/+) animals following clodronate administration. (a) Serum iron parameters in iron supplemented (parenteral iron dextran) th3/+ animals following PBS- or clodronate- liposome treatment. (b) CD71-Ter119 (upper panels) and erythroid (R1 gate) CD44-FSC (lower panels) profiles from the spleen of PBS- or clodronate-treated th3/+ animals supplemented with parenteral iron dextran. (c) Total number of immature CD71highTer119high (C+T+) or mature CD71lowTer119high (C-T+) erythroid cells in the spleens of iron dextran supplemented th3/+ mice treated with PBS- or clodronate-liposomes (from top panels in (b)). (d) Total number of cells in each stage of erythroid differentiation (I to V) based on FSC-CD44 profiles shown in (b). In this experiment animals were subjected to two liposome IV injections at day 0 (100 µl) and day 7 (80 µl). Parenteral iron (20 µg iron dextran) was administered daily by IP route and mice were analyzed at day 8.
Supplementary figure 18. Serum Epo levels in clodronate-treated $Hbb^{th3/+}$ ($th3/+)$ mice. Serum Epo levels in wt control, PBS- or clodronate-treated $th3/+$ mice. Values are shown as the mean ± SEMs of at least 6 mice per group. Serum was collected 1 month from the beginning of the chronic liposome treatment. At this point erythropoietic activity is already markedly decreased in clodronate-treated $th3/+$ animals, compared to PBS $th3/+$ controls.
Supplementary figure 19. Chronic clodronate treatment reduces the total number of erythroid progenitors in the BM and spleen of Hbb\textsuperscript{th3/+} (th3/+) mice. Graphs show the total number of immature CD71\textsuperscript{high}Ter119\textsuperscript{high} (C+T+) or mature CD71\textsuperscript{low}Ter119\textsuperscript{high} (C-T+) erythroid cells in the BM and spleen of th3/+ mice chronically treated with PBS- or clodronate-liposomes. Data represents the quantification of total cells based on profiles shows in figure 4g. The values are presented as means ± SEMs of 3 animals per group. Statistical significance is shown by * P ≤ 0.05, ** P ≤ 0.01.
Supplementary figure 20. Macrophages decrease apoptosis of human erythroblasts. (a) Apoptosis analysis by flow cytometry of human erythroid cells at day 6 of differentiation. Annexin V was used as an apoptotic marker, while 7AAD was used as a late apoptotic/necrotic marker. The left panels represent human erythroblasts cultured alone, while right panels represent human erythroblasts derived from co-cultures with macrophages at day 6 of erythroid differentiation.
Supplementary figure 21. Characterization of differentiation of human erythroblasts in *in vitro* cultures. (a) May-Grunwald Giemsa stain of cytospins prepared from EB before the start of differentiation (Undif.), or after 4 and 8 days of EB differentiation (Dif) in culture with (+Mac) or without (-Mac) macrophages. (b) Flow cytometric characterization of undifferentiated EB, or EB at day 4 and day 8 of differentiation in the presence or absence of macrophages. Forward-scatter/side-scatter profiles and expression of the differentiation markers c-Kit, CD44, Band3 and glycophorin A (GPA) are shown. No clear differences in the morphology or these cell surface marker expression were seen comparing EB cultured in the presence or absence of macrophages.
Supplementary figure 22. Expression of beta1-integrin in human cultures at day 8 of erythroid differentiation. Figure shows two representative profiles of expression of beta1-integrin in EB cultured alone or in the presence of macrophages at day 8 of differentiation. At this time-point, both the percentage of cells expressing beta-1 integrin, as well as β1 integrin levels are increased in EB in co-culture with macrophages.
Supplementary figure 23. Clodronate/PBS liposome administration regiments. (a) Schematic representation of clodronate administration in phlebotomized wt mice. 100 μl of PBS- or clodronate-liposomes were administered IV at day 0, day 4 and day 8. Clod-(d4) group was treated with PBS liposomes at day 0 and then, clodronate liposomes at day 4 and day 8. Phlebotomy was performed at day 0, day 1 and day 2 of experimental procedure, and recovery from anemia was followed for up to day 20 by CBC. For iron-supplemented, *Hfe*-KO and *Hamp*-KO mice a single injection of liposomes was performed at D0. (b) Experimental design of hrEPO and liposome co-administration. 50 units of hrEPO were administered daily to WT mice for up to one week. Liposomes (100 μl) were injected IV at day 0 and day 4. Hematological parameters were followed by CBC at day 0, day 4 and day 8. (c) Clodronate administration regimen for mice affected by Polycythemia vera. Liposomes (200 μl) were administered once a week, starting at day 16 post-BM transplantation (BMT). A total of 4 liposome injections were performed. Hematological parameters were measured by CBC at the time-points indicated. (d) Chronic liposome administration to WT and *Hbbth3/+* mice was performed for up to 12 weeks. Liposomes (50 μl) were administered every two weeks. Hematological parameters were measured in the liposome bye-week, as indicated. Iron-supplementation of *Hbbth3/+* mice was carried out by administration of an iron-rich diet (with 2% carbonyl-iron) (e) or daily IP injections of 20 μg iron-dextran (f). In each experiment two IV injections of liposomes were performed at the indicated times.