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Plenary Paper

RED CELLS, IRON, AND ERYTHROPOIESIS

Genetic iron overload aggravates, and pharmacological iron restriction improves, MDS pathophysiology in a preclinical study

Ada Antypiuk,[1,*] S. Zebulon Vance,[1,*] Richa Sharma,[1,†] Sara Passos,[1,†] Michela Asperti,[1,2] Shobana Navaneethabalakrishan,[1] Franz Dürrenberger,[3] Vania Manolova,[3] and Francesca Vinchi[1,4]

[1]Iron Research Laboratory, Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY; [2]Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy; [3]CSL Research, Zurich, Switzerland; and [4]Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY

KEY POINTS

- **Iron restriction in MDS mice improves erythropoiesis, preserves the HSPC pool, limits myeloid expansion, and delays leukemic transformation.**
- **Combining iron restriction and erythroid maturation drugs shows superior improvement of erythropoiesis and disease-modifying potential in MDS.**

Although iron overload is a common feature in myelodysplastic syndromes (MDS), it remains unclear how iron excess is detrimental for disease pathophysiology. Taking advantage of complementary approaches, we analyzed the impact of iron overload and restriction achieved through genetic activation of ferroportin (FPN) via the C326S mutation (FPN^{C326S}) and pharmacologic inhibition (vamifeport) of the iron exporter FPN, respectively, in a MDS mouse model. Although FPN^{C326S}-induced iron overload did not significantly improve the late stages of erythroid maturation, vamifeport-mediated iron restriction ameliorated anemia and red blood cell maturation in MDS mice, through the reduction of oxidative stress and apoptosis in erythroid progenitors. Iron overload aggravated, and restriction alleviated, reactive oxygen species formation, DNA damage, and cell death in hematopoietic stem and progenitor cells (HSPCs), resulting in altered cell survival and quality. Finally, myeloid bias, indicated by expanded bone marrow myeloid progenitors and circulating immature myeloid blasts, was exacerbated by iron excess and attenuated by iron restriction. Overall, vamifeport treatment resulted in improved anemia and significant survival increment in MDS mice. Interestingly, the combined therapy with vamifeport and the erythroid maturation agent luspatercept has superior effect in improving anemia and myeloid bias as compared with single treatments and offers additive beneficial effects in MDS. Our results prove, to our knowledge, for the first time in a preclinical model, that iron plays a pathologic role in transfusion-independent MDS. This is likely aggravated by transfusional iron overload, as suggested by observations in the FPN^{C326S}MDS model. Ultimately, the beneficial effects of pharmacologic FPN inhibition uncovers the therapeutic potential of early prevention of iron toxicity in transfusion-independent MDS.

Introduction

Patients with myelodysplastic syndromes (MDS), a heterogenous group of clonal myeloid neoplasms, are prone to develop iron overload as consequence of ineffective erythropoiesis, which hallmarks the disease, and transfusion therapy, which is required to correct the underlying anemia. In MDS, although the genetic defects prevent adequate mature red blood cell (RBC) production, the ensuing hypoxia induces erythropoietin (EPO) release and erythroid progenitor expansion in an unsuccessful attempt to recover the anemia. This process increases the demand for iron, which is absorbed in higher amount from duodenal enterocytes through the iron exporter ferroportin (FPN).[1-3] This is mediated by erythroid-driven suppression of the iron regulatory hormone hepcidin, which controls systemic iron influx from enterocytes and macrophages by binding and inducing the degradation of FPN.[1-4] The recently discovered erythroid hormone erythroferrone and hepatokine fibrinogen-like 1 act as hepcidin suppressors by scavenging bone morphogenetic proteins that control hepcidin induction.[5,6] Enhanced iron absorption and limited iron use result in elevated transferrin saturation, leading to formation of non–transferrin-bound iron (NTBI) and its accumulation in tissue, causing iron overload in MDS.[1-3]

Speculations about iron toxicity in MDS are often transposed from observations in transfusion-dependent β-thalassemia without substantial evidence. In this regard, controversies arise

from the fact that elderly patients with MDS are exposed to iron overload later in life, for a shorter period, and hence to an overall lower iron amount than those receiving life-long transfusion therapy.[1,2,7-10] Furthermore, iron-related complications often overlap and might aggravate age-related clinical features in patients with MDS, which prevents discriminating the additional contribution of iron overload to morbidities and mortality. Although evidence of the detrimental effects of iron in MDS have been obtained almost exclusively by studying MDS mouse models in which iron overload was "artificially" induced through iron injections mimicking acute rather than chronic iron toxicities, a clear understanding of the impact of pathophysiologic iron excess in preclinical MDS models is lacking and whether/how iron excess aggravates bone marrow (BM) failure has remained understudied, with limited preclinical studies and randomized prospective trials.[1,7,9-13]

Importantly, because of the scarce treatment options available for MDS, the identification of areas of modifiable risk including iron-induced toxicity, may offer novel therapeutic opportunities and improve outcomes for patients with MDS. The erythroid maturating agent luspatercept has recently been approved for the treatment of MDS with SF3B1 mutation failing erythroid stimulating agents, and then labeled by the US Food and Drug Administration as first-line therapy for low-risk MDS with/without SF3B1 mutation because of its beneficial effect on anemia and reduction of transfusion burden.[14-17] However, the limited action of luspatercept on disease allelic burden calls for better or combined therapeutic strategies providing further benefit in MDS. In this context, to establish a clear role for iron dyshomeostasis and toxicity as well as the benefit of novel iron restrictive approaches in MDS is relevant and compelling.

Taking advantage of complementary approaches to modulate iron levels in preclinical MDS mouse models, we sought to address the impact of iron on MDS pathophysiology. Here, we show that iron overload aggravates MDS pathophysiology by worsening dysplastic hematopoiesis and erythropoiesis and boosting myeloid expansion through NTBI-mediated exacerbation of MDS features, such as excessive reactive oxygen species (ROS) production, hematopoietic stem and progenitor cell (HSPC) exhaustion, myeloid bias, and genotoxic stress. By contrast, decreasing systemic iron levels and NTBI formation by the clinical stage FPN inhibitor vamifeport (VIT)[18-21] improves anemia, limits myeloid expansion, and delays MDS progression to acute myeloid leukemia (AML), with significant survival benefit in MDS mice. Finally, we proved that combination therapy with VIT and luspatercept shows superior effects in improving anemia and myeloid bias as compared with single treatments.

Methods

Mouse models and treatments

Wild-type, NUP98-HOXD13 MDS,[22,23] FPN^{C326S}, and MDS FPN^{C326S} mice[24,25] on a C57BL/6 genetic background were bred and housed in the laboratory animals research services at the Lindsley F. Kimball Research Institute, New York Blood Center. Experiments were approved by and conducted in compliance with the guidelines of the institutional animal care and use committee of the New York Blood Center.

Flow cytometry analysis

Flow cytometry was performed as previously reported.[26]

Serum iron, NTBI, and tissue iron measurement

Iron measurements were performed using the bathophenanthroline colorimetric method as previously described.[25]

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Comparisons between 2 groups were performed with 2-sided Welch *t* tests, and among ≥3 groups with 1-way analysis of variance followed by Bonferroni posttest, using GraphPad Prism version 8. $P < .05$ was considered significant.

Details on materials and methods are included in supplemental Data, available on the *Blood* website.

Results

FPN activation exacerbates, and FPN inhibition improves, the iron phenotype of MDS mice

The impact of iron in MDS was investigated in NUP98-HOXD13 MDS mice[22,23] by applying complementary approaches aimed at targeting FPN: iron overload and restriction were achieved through genetic activation of FPN via the C326S mutation (FPN^{C326S}), and pharmacological inhibition of FPN via the small-molecule VIT[18-21] in MDS mice, respectively. Mice were analyzed at 6 months of age (Figure 1A-B). MDS mice showed an iron phenotype at steady state, as indicated by elevated serum iron and NTBI levels, increased tissue iron content (liver and kidney), and reduced hepcidin levels (Figure 1C-G; supplemental Figure 1). Most of these parameters were enhanced and reduced by FPN activation and inhibition, respectively (Figure 1C-G). Spleen iron content was slightly increased in MDS mice, reduced by FPN activation and further elevated by FPN inhibition (Figure 1Eiii,Fiii; supplemental Figure 2). Although hepcidin was induced by iron overload in presence of the FPN^{C326S} mutation, its levels together with the hepcidin/liver iron ratio were lower in MDS and MDS FPN^{C326S} mice compared with the respective wild-type and FPN^{C326S} counterparts (Figure 1G), suggesting inadequate hepcidin production compared with iron burden.

Overall, these observations indicate that MDS mice develop iron overload at steady state because of hepcidin suppression. Targeting FPN export function modulates the iron status in MDS animals by altering systemic iron influx, thus providing optimal complementary models of iron overload and restriction to dissect iron impact on MDS pathophysiology and progression.

Iron restriction improves ineffective erythropoiesis and anemia in MDS mice

To evaluate the impact of altered iron levels on anemia in MDS, we analyzed complete blood count and BM and splenic erythropoiesis in the MDS mouse models. At 6 months of age, MDS mice presented clear signs of ineffective hematopoiesis as suggested by the macrocytic anemia, reticulocytosis, leukopenia, and thrombocytopenia (Figure 2A-B; supplemental Figure 3A-B).

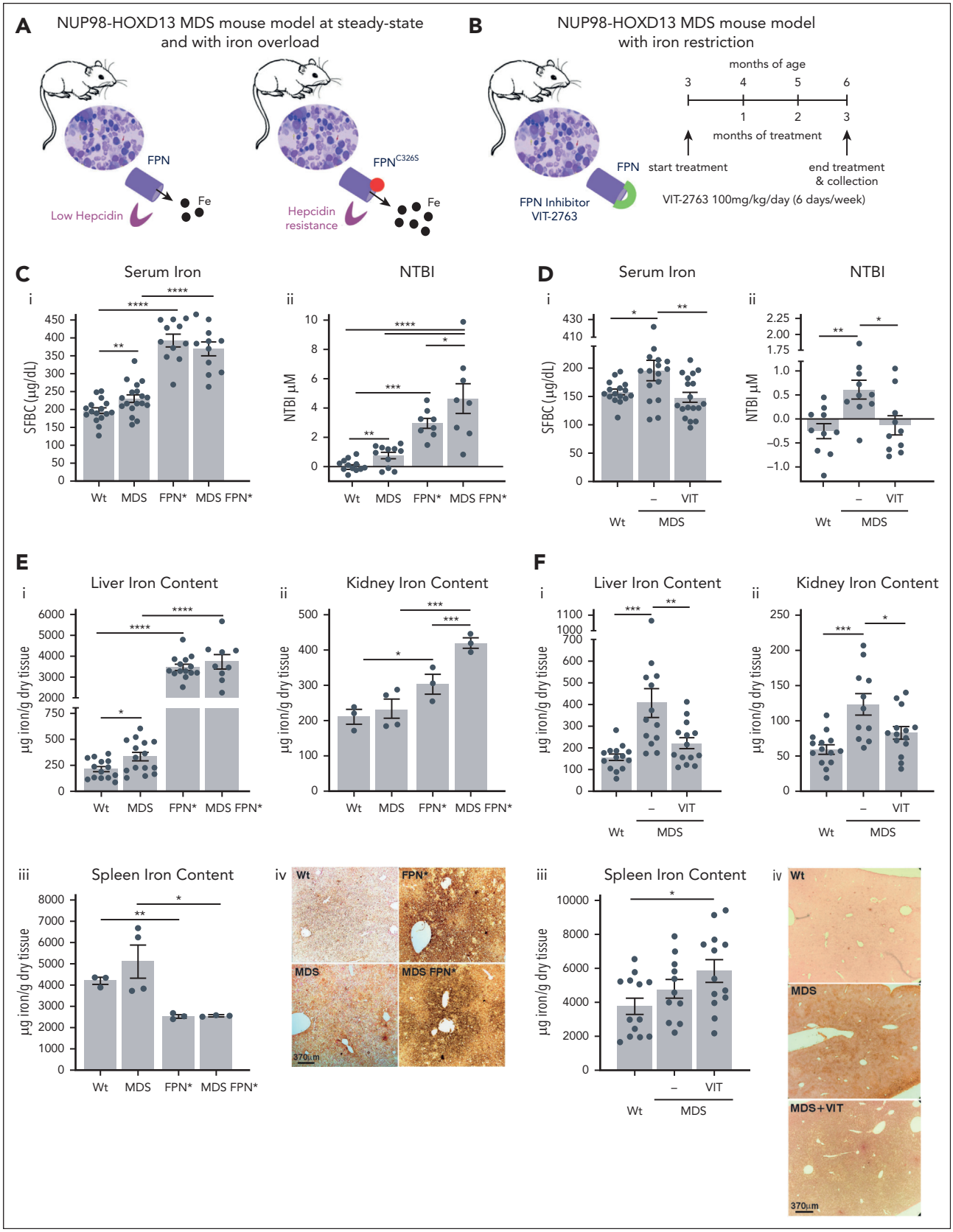


Figure 1. FPN activation and inhibition aggravates and attenuates the iron phenotype of MDS mice, respectively. MDS mouse models of iron overload (A) and iron restriction (B) used in these studies. (C-D) Serum iron (i), NTBI (ii); (E-F) tissue iron content of the liver (i), kidney (ii), and spleen (iii), and representative Perls' staining for iron on liver sections (iv) of wild-type, MDS, FPN*, and MDS FPN* mice, and wild-type and MDS mice receiving vehicle or VIT for 3 months. (G) Serum hepcidin (i) and serum hepcidin/liver iron ratio (ii) in wild-type, MDS, FPN*, and MDS FPN* mice. Data shown are average of at least 3 independent experiments. Values represent mean ± SEM. Statistical analysis was performed by comparing ≥3 groups with 1-way analysis of variance (ANOVA) followed by Bonferroni posttest. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

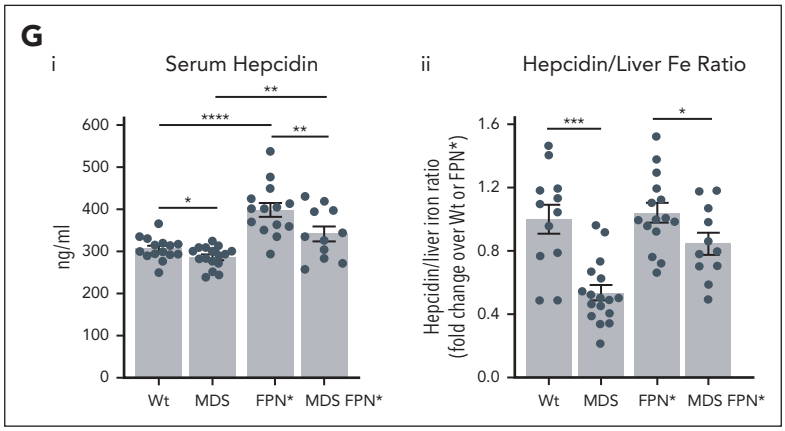


Figure 1 (continued)

The increased iron availability likely accounts for the slight increase in hemoglobin (Hb) levels observed in FPN^{C326S} and MDS FPN^{C326S} mice compared with the wild-type and MDS counterparts (Figure 2Ai). However, MDS FPN^{C326S} mice failed to show a significant improvement in Hb and RBC count compared with control FPN^{C326S} mice and presented similar differences in RBC and complete blood count parameters as observed between MDS and wild-type mice, with most parameters remaining unchanged compared with MDS mice (Figure 2Ai-vi; supplemental Figure 3Ai,Bi). Overall, this suggests that increased iron availability is not sufficient to correct the underlying anemia in MDS. By contrast, iron restriction by VIT improved anemia in MDS mice, as indicated by the higher Hb and hematocrit levels, increased RBC number, reduced mean cellular volume and mean cellular Hb, reticulocytosis, and improved thrombocytopenia (Figure 2Bi-vi; supplemental Figure 3Aii,Bii). VIT beneficial effects were observed after 2 months of VIT treatment and lasted for 3 months in MDS mice (Figure 2Ci-v,Di-iii). Taken together, these data demonstrate that iron restriction but not iron excess ameliorates anemia and thrombocytopenia in MDS.

BM cells positive for the erythroid marker Ter119^{+} were reduced in MDS mice compared with wild-type counterparts, along with increased expression of the transferrin receptor CD71 (Figure 3Ai-iii,Bi-iii). Ineffective erythropoiesis in MDS was associated with an expansion of immature Ter119^{+}CD71^{+} and decrease of more mature Ter119^{+}CD71$^{int/low}$ erythroid precursors (Figure 3Aiii,iv,Biii,iv). Defective RBC maturation was suggested by the expansion of the Ter119^{+}CD44$^{+/int}$ populations of proerythroblasts, basophilic, and polychromatic erythroblasts, with a concomitant decrease in Ter119^{+}CD44^{-} mature RBCs (Figure 3Ci-vi,Di-vi). MDS FPN^{C326S} mice did not show a significantly higher number of Ter119^{+}CD71^{low} cells as well as Ter119 CD44^{-} RBCs compared with MDS mice, despite the reduction in immature Ter119^{+}CD71^{+} erythroid precursors and Ter119^{+}CD44$^{+/int}$ populations (Figure 3Aiii,iv,Ci-vi). This indicated that, although increased iron availability stimulates erythroid differentiation by promoting the first stages of erythroblast maturation, it does not improve the defect of terminal differentiation imposed by MDS. EPO levels, which were strongly elevated in MDS mice, were reduced by increased iron in MDS FPN^{C326S} mice, in agreement with the slight Hb increase and early erythroblast reduction (Figure 2Ai,Ci-iii; supplemental Figure 4A). Importantly, BM Ter119^{+} cells showed increased ROS levels and phosphatidylserine (PS) exposure in MDS mice (Figure 3E-H). This was further elevated in iron-loaded MDS FPN^{C326S} mice, suggesting that iron aggravates ROS production and apoptosis in erythroid precursors (Figure 3E,G; supplemental Figure 5), counteracting the erythroid differentiation effect due to its increased availability.

VIT-treated MDS mice showed an overall increase in BM Ter119^{+} erythroid cells compared with untreated mice, with reduced number of immature Ter119^{+}CD71^{+} and increased number of mature Ter119^{+}CD71$^{int/low}$ erythroid cells (Figure 3Bi-iv). Iron restriction in MDS mice decreased the expansion of Ter119^{+}CD44$^{+/int}$ proerythroblasts and basophilic and polychromatic erythroblasts, and enhanced Ter119^{+}CD44^{-} RBCs, indicating a more effective erythropoiesis (Figure 3Di-vi). Accordingly, EPO levels were reduced by VIT treatment in MDS mice (supplemental Figure 4B). This was associated with reduced ROS production and decreased apoptosis in BM Ter119^{+} erythroid cells (Figure 3F,H; supplemental Figures 6 and 7), as well as reduced mitochondria retention in BM Ter119^{+} cells, circulating reticulocytes, and RBCs (supplemental Figure 8), suggesting that iron restriction improves erythropoiesis at least in part by limiting erythroblast ROS production and apoptosis. VIT normalized the labile iron pool (LIP) and reduced ROS production throughout most of the erythroid populations in MDS mice (supplemental Figure 7A-B). Extramedullary erythropoiesis, indicated by increased splenomegaly and splenic erythropoiesis output, was reduced by VIT treatment and slightly increased by the FPN^{C326S} mutation in MDS mice (supplemental Figures 9-11). Finally, improved anemia and erythropoiesis were recapitulated in MDS mice treated with human transferrin, confirming that iron restriction, obtained either via reduced iron absorption or iron scavenging, is of benefit for erythroid maturation (supplemental Figure 12).

Overall, these results demonstrate that iron overload is detrimental to dysplastic erythropoiesis, whereas limiting iron availability provides benefit to MDS-related anemia.

Iron overload aggravates and iron restriction limits HSC exhaustion and MP expansion in MDS mice

Taking into account that MDS is a disease originating in HSPCs, we evaluated the impact of iron on BM HSPCs. In MDS mice, HSPCs were exhausted and skewed toward the myeloid lineage, as indicated by the depletion of the BM pool of

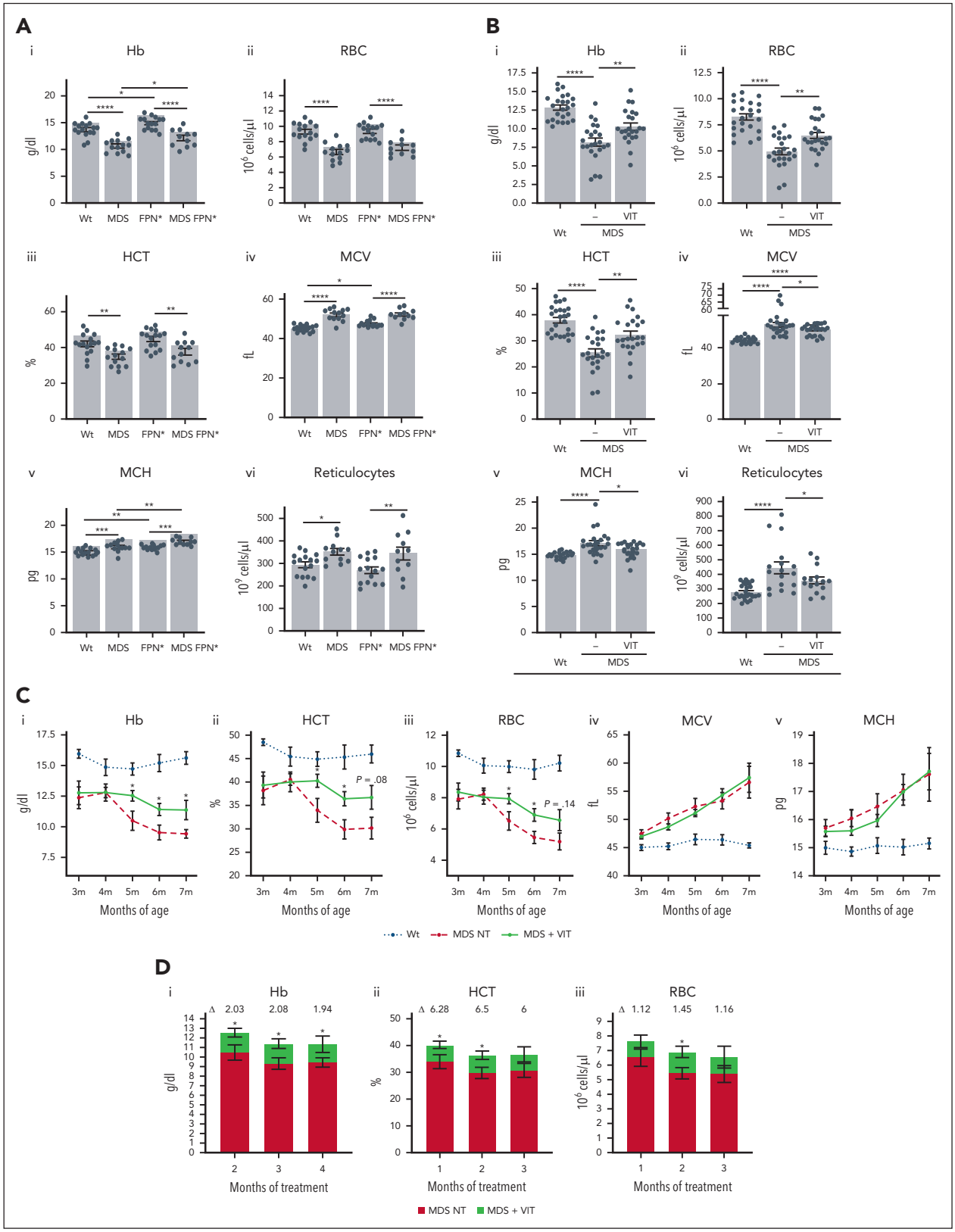
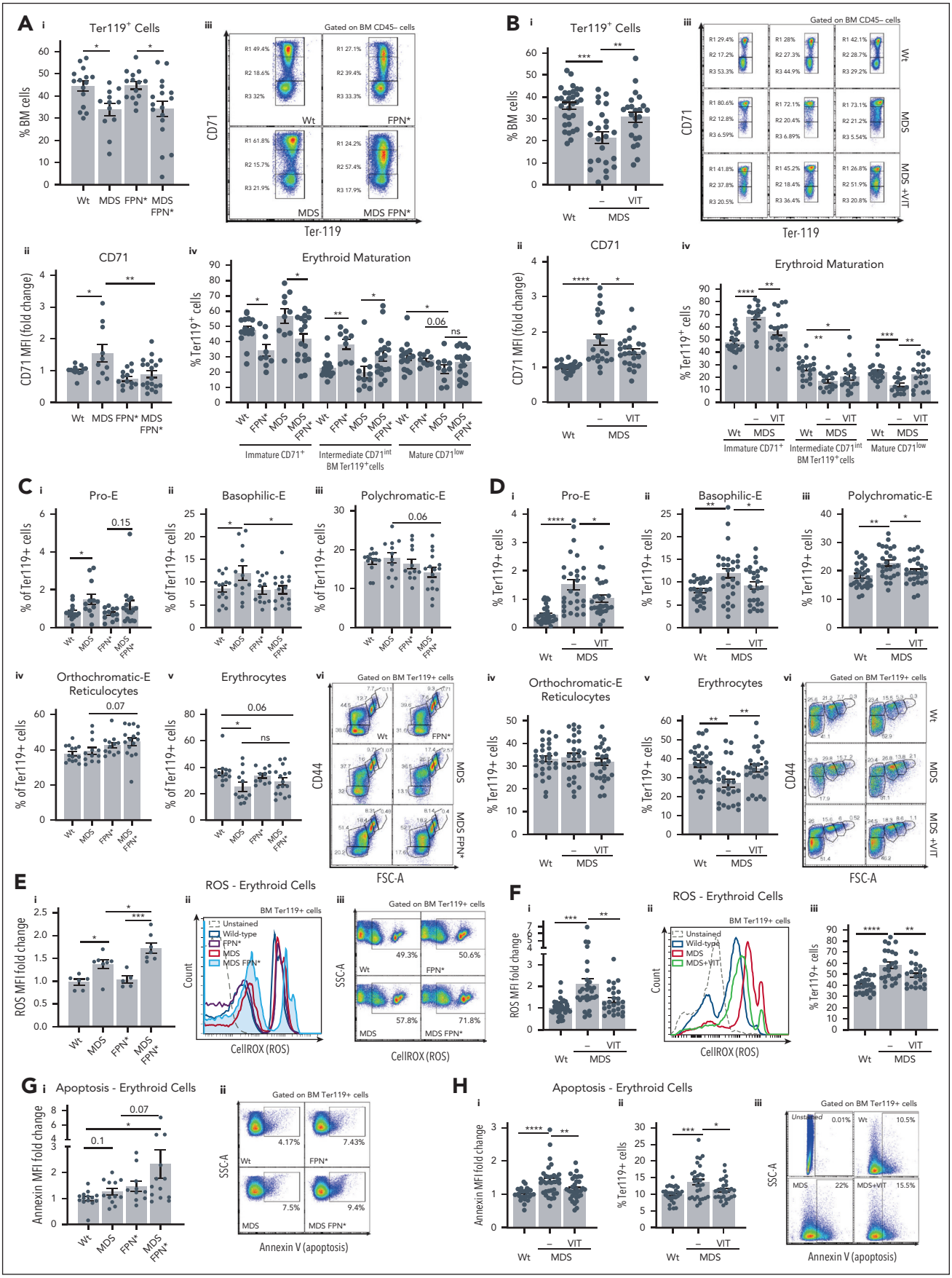


Figure 2. Anemia is improved by iron restriction but not iron overload in MDS mice. (A-B) Hb (i), RBC (ii), hematocrit (HCT) (iii), mean cellular volume (MCV; iv), mean cellular Hb (MCH; v), and reticulocytes (vi) in wild-type, MDS, FPN*, and MDS FPN* mice, and wild-type and MDS mice receiving vehicle or VIT for 3 months. (C) Time-course of Hb (i), HCT (ii), RBC (iii), MCV (iv), and MCH (v) in wild-type and MDS mice receiving vehicle or VIT from 3 months of age onward. (D) Hb levels (i), HCT (ii), and RBC (iii) in MDS mice receiving vehicle or VIT for 2, 3, and 4 months (corresponding to 5, 6, and 7 months of age). Hb, HCT, and RBC increase (D) is shown in VIT-treated vs control MDS mice. On average, Hb was improved by ~2 g/dL, HCT by ~6%, and RBCs by >1 × 10^6 cells per mL blood in VIT-treated MDS mice from 5 through 7 months of age. Data shown are average of at least 3 independent experiments. Values represent mean ± SEM. Statistical analysis was performed by comparing ≥3 groups with 1-way ANOVA followed by Bonferroni posttest. *P < .05; **P < .01; ***P < .001; ****P < .0001.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Gated on BM Ter119+ cells
Unstained
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Annexin V (apoptosis)

Figure 3.

$Lin^{-}cKit^{+}Sca\text{-}1^{+}$ (LSK) HSPCs and expansion of $Lin^{-}cKit^{+}$ (LK) myeloid progenitors (MPs) compared with wild-type mice (Figure 4A-B). Iron overload in MDS FPN^{C326S} mice left the population of LSK HSPCs depleted and triggered a further expansion of the LK MP pool (Figure 4Ai-iii). By contrast, iron restriction by VIT partially rescued the loss of LSK HSPCs and limited LK MP expansion in MDS mice (Figure 4Bi-iii).

LSK cells in the MDS BM showed a more ample LIP (Figure 4C-D), together with increased ROS levels and membrane PS exposure, as well as higher percentage of ROS^{+} and PS^{+} LSK cells, which were further aggravated in MDS FPN^{C326S} mice and decreased in VIT-treated MDS mice (Figure 4C-H; supplemental Figure 13A-F). Similar changes were observed in LK MPs (supplemental Figures 14 and 15). Overall, this suggests that the iron status modulates HSPC loss through the induction of ROS formation and apoptotic cell death.

Chromosome instability, DNA damage, and defective repair are involved in MDS progression from low- to high-risk MDS and AML. We monitored the presence of DNA double-strand breaks in HSPCs by staining phosphorylated histone γ-H2AX as marker of DNA damage and instability. LSK HSPCs presented more double-strand breaks in the MDS than wild-type BM, as shown by the higher cellular levels and percentage of $\gamma\text{-H2AX}^{+}$ LSK cells (Figure 4I-J). DNA damage in MDS LSK cells was aggravated by the FPN^{C326S} mutation and ameliorated by VIT treatment, indicating that iron overload promotes, whereas iron restriction limits, genetic instability in MDS (Figure 4I-J; supplemental Figure 13G-H).

Taken together, these results demonstrate that iron, by triggering HSC exhaustion and MP expansion, affects the HSPC pool and promotes clonal evolution in MDS.

Iron overload exacerbates, and iron restriction limits, myeloid skewing in MDS mice

To assess the impact of iron on myeloid bias we analyzed myeloid cells in the BM and peripheral blood of the MDS mouse models. Compared with wild-type controls, MDS mice showed a higher percentage of BM $CD45^{+}$ immune cells over BM total and mononuclear cells (Figure 5A-B). BM $CD11b^{+}$ myeloid cells were increased, whereas $CD11b^{-}$ lymphoid cells were reduced (Figure 5C-D; supplemental Figure 16), suggesting myeloid expansion and defective lymphoid lineage commitment. Importantly, MDS FPN^{C326S} mice showed further expansion of $CD45^{+}$ cells and $CD11b^{+}$ myeloid cells and reduced lymphoid population in the BM compared with control MDS animals (Figure 5A,C; supplemental Figure 16A). By contrast, VIT-treated MDS mice showed a lower percentage of BM $CD45^{+}$ cells and $CD11b^{+}$ myeloid cells and a higher percentage of $CD11b^{-}$ lymphoid cells compared with untreated MDS mice (Figure 5B,D; supplemental Figure 16B). The enlarged myeloid pool in MDS mice positive for the monocyte/granulocyte marker Gr-1, with high monocytic $Ly6C^{+}$ cell percentage, was further expanded in MDS FPN^{C326S} mice and reduced in VIT-treated MDS mice (Figure 5E-H).

Overall, these data demonstrate that iron overload aggravates, whereas iron restriction reduces, myeloid expansion in the MDS marrow, thus indicating that iron is a modifier of myeloid skewing.

Combining iron restrictive and erythroid maturating therapies has a superior effect on the improvement of MDS pathophysiology compared with single treatments

Because of the lack of disease-modifying therapy by the erythroid maturation agent luspatercept,[14,15] we sought to address whether its combination with the FPN inhibitor VIT could provide further benefit in MDS.

The combined therapy further improved anemia compared with the treatments with either drug alone, as suggested by the higher Hb levels, hematocrit and RBC count, and decreased early erythroid precursors, EPO levels, and splenomegaly, signs of more effective erythropoiesis (Figure 6ABi-iii; supplemental Figures 4B, 9B, 16, and 18). Interestingly, luspatercept, either alone or combined, caused a more significant increase of $Ter119^{+}$ BM cells than single VIT treatment (supplemental Figure 18A), whereas VIT, either alone or combined, improved early and late erythroblasts more significantly than single luspatercept treatment (Figure 6Bi-ii; supplemental Figure 18B). Thus, the superior effect of the combined treatment on anemia is driven by both the higher number and improved quality of erythroid cells triggered by the 2 drugs. VIT treatment reduced erythroid ROS production and apoptosis, and prevented the depletion of the HSPC pool as well as LK MP expansion as a single and combined therapy with luspatercept (Figure 6Biv-vi,C-D; supplemental Figures 19 and 20).

Importantly, VIT but not luspatercept significantly altered myeloid expansion in MDS. $CD45^{+}$ immune cell expansion and myeloid bias, monitored as percentage of $CD11b^{+}$ and $CD11b^{+}$ $Gr1^{+}$ myeloid cells in the BM, were attenuated in MDS mice treated with VIT alone and in combination with luspatercept (Figure 6E; supplemental Figure 21).

These results show that VIT therapy combined with luspatercept further improved anemia compared with single treatments, and maintained myeloid disease-modifying activity, having an overall superior effect on improving MDS pathophysiology.

Iron overload reduces, and iron restriction prolongs, the survival of MDS mice

To evaluate whether the effect of altered systemic iron levels on myeloid bias/expansion reflected on MDS-to-AML evolution, we assessed the presence of myeloid blasts expressing the stem cell marker cKit, white blood cell count, and survival in MDS models. The percentage of BM $cKit^{+}$ total cells and $cKit^{+}$ $CD11b^{+}$ myeloblasts was elevated at steady-state and upon luspatercept

Figure 3. Erythropoiesis is worsened by iron overload and ameliorated by iron restriction in MDS mice. (A-B) Erythroid $Ter119^{+}$ cells expressed as percentage of total BM cells (i), CD71 expression in BM $Ter119^{+}$ cells (ii), representative dot plot of BM $Ter119^{+}$ cells (iii), and analysis of erythroid maturation by CD71 loss, showing the percentage of immature $CD71^{high/int}$ and mature $CD71^{low}$ $Ter119^{+}$ cells (iv). (C-D) Analysis (i-v) and representative dot plot (vi) of BM erythroid maturation by CD44 loss, showing the percentage of proerythroblasts, basophilic, polychromatic and orthochromatic erythroblasts, reticulocytes, and erythrocytes. (E-F) Flow cytometry analysis of ROS production and (G-H) apoptosis in BM $Ter119^{+}$ cells, expressed as mean fluorescence intensity (MFI) fold change (i) and/or percentage positive cells (iii), and representative histograms (ii) and/or dot plots (iii), in wild-type, MDS, FPN*, and MDS FPN* mice and wild-type and MDS mice receiving vehicle or VIT for 3 months. Data shown are average of at least 3 independent experiments. Values represent mean ± SEM. Statistical analysis was performed by comparing ≥3 groups with 1-way ANOVA followed by Bonferroni posttest. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

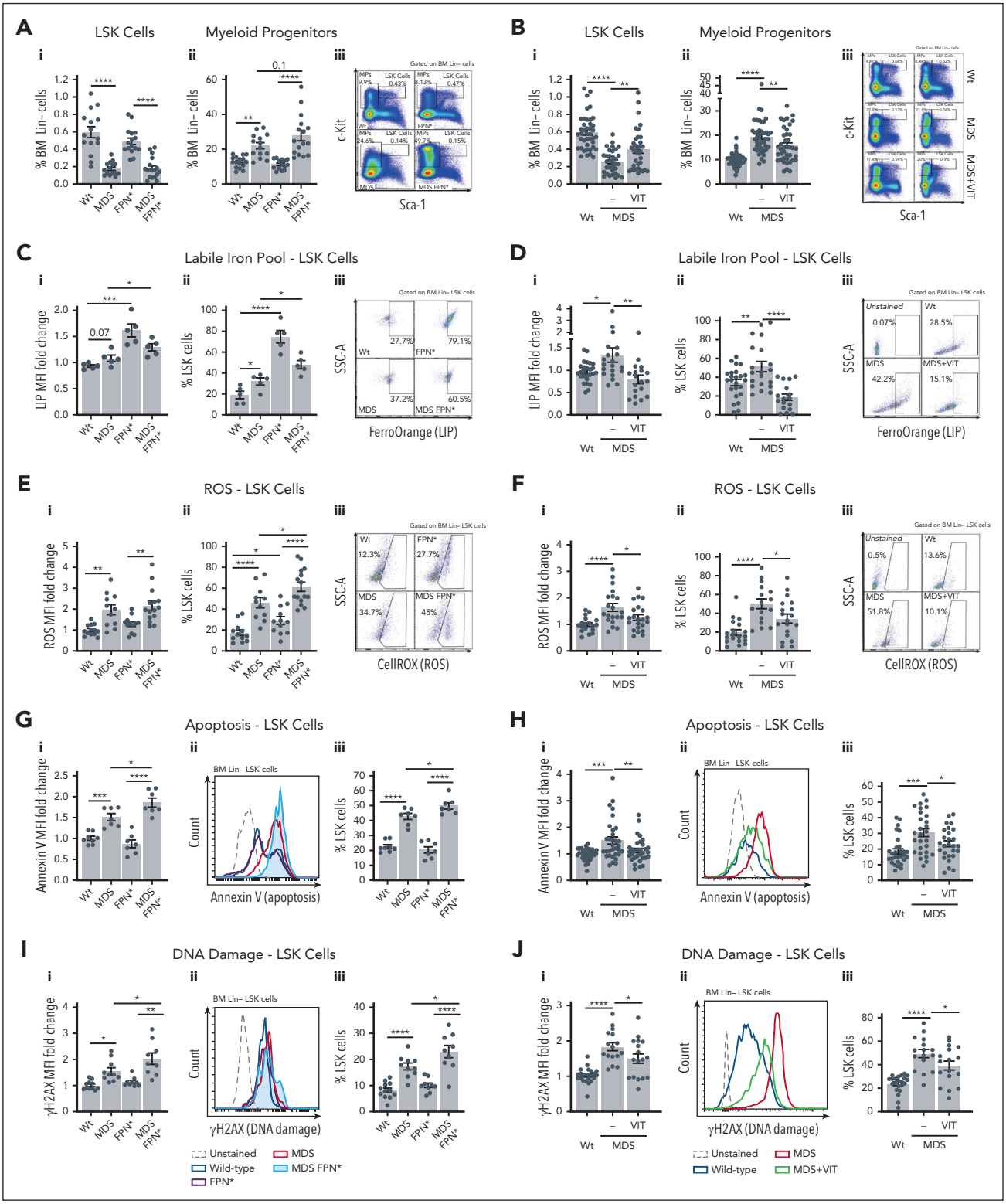


Figure 4. The BM stem cell pool is improved by iron restriction. (A-B) LSK HSPCs (i) and LK MPs (ii) expressed as percentage of BM Lin$^-$ cells; (C-D) Flow cytometry analysis of LIP content, (E-F) ROS production, (G-H) apoptosis and (I-J) DNA damage, expressed as MFI fold change (i) and/or percentage positive cells (ii/iii), in BM LSK cells of wild-type, MDS, FPN*, and MDS FPN* mice, and wild-type and MDS mice receiving vehicle or VIT for 3 months. Representative histograms and/or dot plots are shown (ii/iii). Data shown are average of at least 3 independent experiments. Values represent mean ± SEM. Statistical analysis was performed by comparing ≥3 groups with 1-way ANOVA followed by Bonferroni posttest. *P < .05; **P < .01; ***P < .001; ****P < .0001.

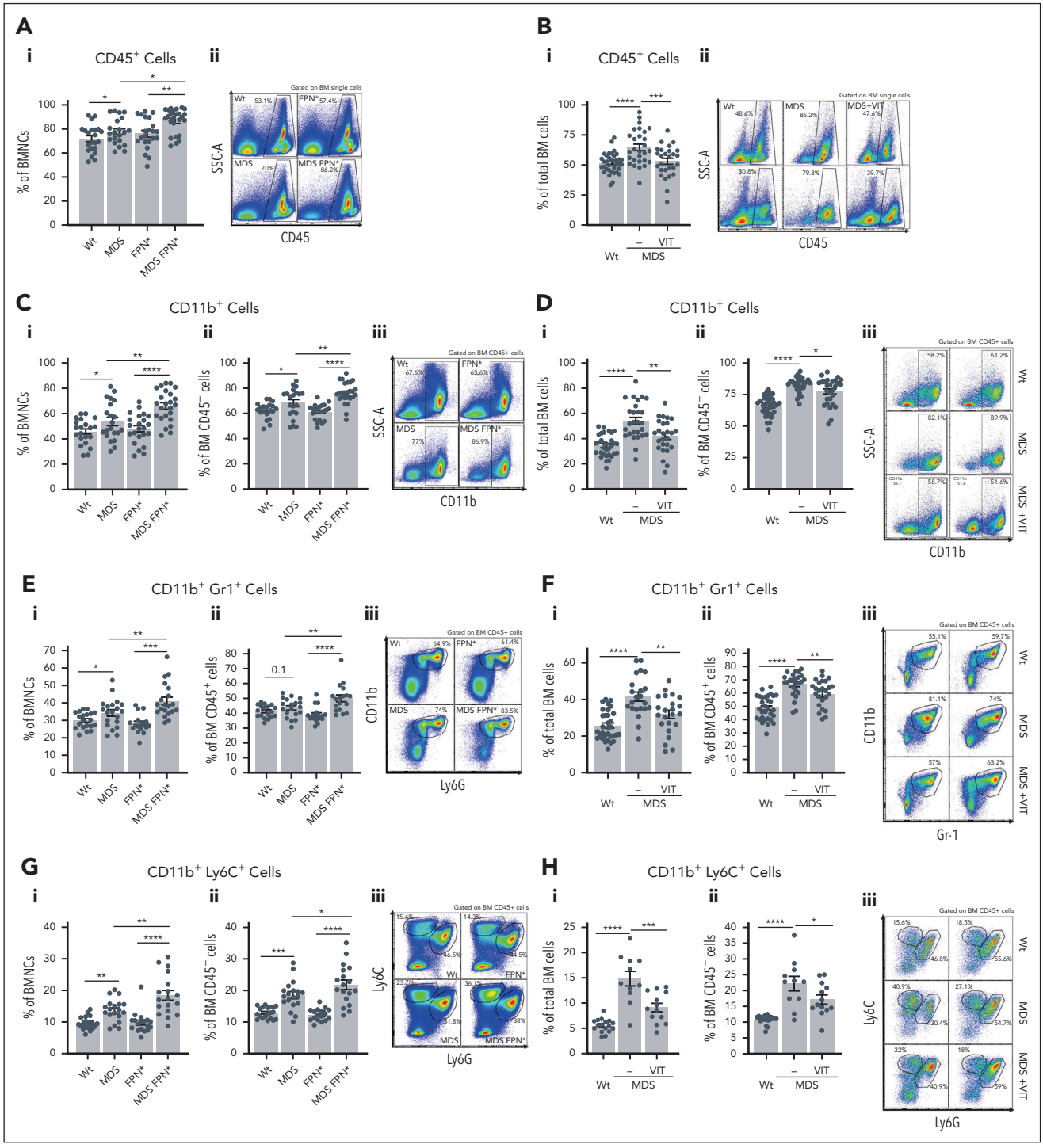
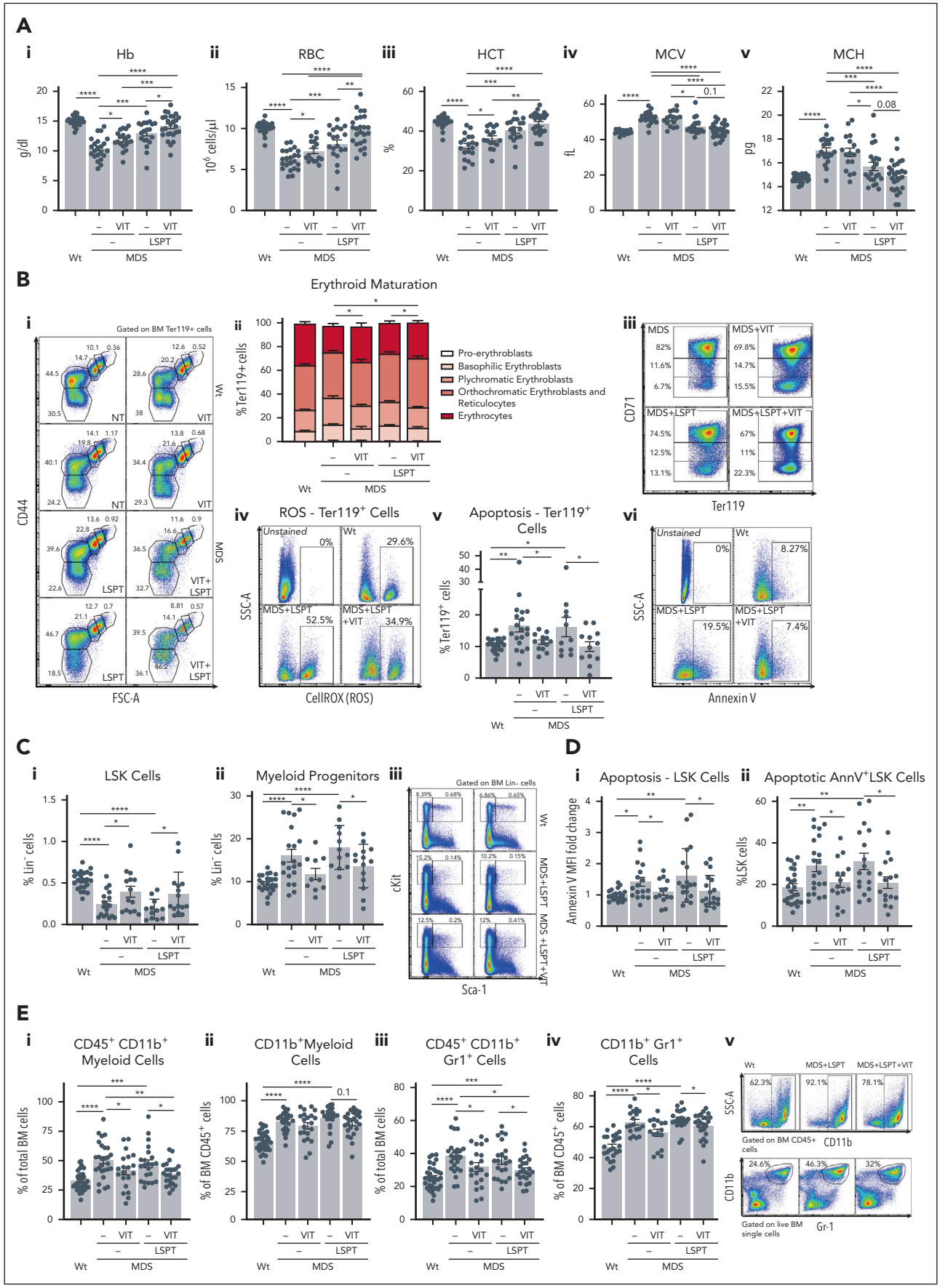


Figure 5. Myeloid skewing and expansion are exacerbated by iron overload and ameliorated by iron restriction in MDS mice. (A-B) Percentage of CD45$^+$, (C-D) CD11b$^+$ myeloid cells, (E-F) CD11b$^+$ Gr-1$^+$ myeloid cells, and (G-H) CD11b$^+$ Ly6C$^+$ monocytic cells in the BM of wild-type, MDS, FPN*, and MDS FPN* mice, and wild-type and MDS mice receiving vehicle or VIT for 3 months. Cell percentage is expressed over BM mononuclear cells for the panels A,C,E,G or BM total cells for the panels B,D,F,H (i), as well as over CD45$^+$ immune cells for the panels A-H (ii). Representative dot plots are shown (ii/iii). Data shown are average of at least 3 independent experiments. Values represent mean ± SEM. Statistical analysis was performed by comparing ≥3 groups with 1-way ANOVA followed by Bonferroni posttest. *P < .05; **P < .01; ***P < .001; ****P < .0001.

treatment, and reduced by iron restriction in MDS mice (Figure 7Ai-iii). Consistent with observations in the BM, the percentage of total CD11$^+$ Gr-1$^+$ myeloid cells and immature cKit$^+$ Gr-1$^+$ myeloblasts was increased in the peripheral blood of MDS animals, both at steady-state and upon luspatercept treatment, and decreased by VIT treatment, alone and as combination (Figure 7Aiv-vii). In most MDS mice, a sharp rise in circulating white blood cells, monocytes, and neutrophils occurred from 8 months of age onward, suggesting MDS-to-AML progression, and was significantly attenuated/delayed by VIT treatment (Figure 7Bi-iii). While 50% of MDS mice died by 250 days, VIT-treated MDS mice showed a significant survival increase, with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Orthochromatic Erythroblasts and Reticulocytes
Erythrocytes
iii
MDS
MDS+VIT
MDS+LSPT
MDS+LSPT+VIT
CD71
Ter119
iv ROS - Ter119+ Cells
Unstained
Wt
MDS+LSPT
MDS+LSPT +VIT
SSC-A
CellROX (ROS)
v Apoptosis - Ter119+ Cells
% Ter119+ cells
vi
Annexin V
C
i LSK Cells
% Lin- cells
ii Myeloid Progenitors
iii
Gated on BM Lin- cells
cKit
Sca-1
D
i Apoptosis - LSK Cells
Annexin V MFI fold change
ii Apoptotic AnnV+LSK Cells
%LSK cells
E
i CD45+ CD11b+ Myeloid Cells
% of total BM cells
ii CD11b+Myeloid Cells
% of BM CD45+ cells
iii CD45+ CD11b+ Gr1+ Cells
iv CD11b+ Gr1+ Cells
v
Gated on BM CD45+ cells
CD11b
Gated on live BM single cells
Gr-1

Figure 6.

50% mice still alive at day 310 (Figure 7C). Although all MDS mice were dead by 400 days, we identified an early time window between day 100 and 300 when MDS mice clearly benefited from VIT-induced iron restriction. By contrast, a higher percentage of MDS FPN^{C326S} mice died within 6 to 8 months of age compared with control MDS mice (Figure 7D). These observations suggest that iron overload accelerates, whereas iron restriction delays, MDS-to-AML transformation.

Discussion

These studies show, to our knowledge for the first time, that an iron overload phenotype, featuring hepcidin suppression, accelerated iron absorption, and NTBI formation, develops in a preclinical MDS mouse model, and is aggravated by further iron loading and reduced by pharmacological iron restriction. We manipulated iron levels in MDS mice by activating FPN through the addition of the gain-of-function C326S mutation in FPN, which caused unrestricted iron absorption,[24,25] or by inhibiting FPN through the administration of the oral inhibitor VIT, which limited iron absorption and recycling.[18-21] Through the characterization of these preclinical models, this work addresses the longstanding controversial issue of the role of iron in MDS, and show that iron excess, through a multifactorial toxic action, aggravates MDS pathophysiology and promotes leukemic transformation.[1,2,8] Ultimately, this work crucially proves the preventive benefits of iron restriction against MDS evolution.[1,2]

Ineffective erythropoiesis is a major driver of enhanced iron absorption in transfusion-independent MDS. In agreement with previous observations in patients with MDS, our data show that hepcidin is suppressed in MDS, both at steady-state and when ample iron stores are available, supporting the inappropriateness of hepcidin levels and the dominant role of the erythroid activity on hepcidin regulation over that of body iron.[4,27] Consequently, iron accumulation in MDS is self-maintained in a "vicious cycle," because of hepcidin inhibition by the expanded BM activity and inadequate iron use by ineffective erythropoiesis.

Iron might affect erythropoiesis by inducing dyserythropoiesis and defects in erythroid maturation.[11] Iron excess suppresses HSPC differentiation toward the erythroid lineage, reduces the formation of erythroid burst-forming unit colonies, induces the development of dysplastic immature erythroblasts, impairs terminal differentiation, and promotes apoptosis of erythroblasts by elevating intracellular ROS.[11,12,28-30] Although hemichromes mediate iron toxicity in β-thalassemia erythroblasts, they are absent in MDS dysplastic erythroblasts.[31] Our data suggest that increased NTBI sensitivity rather accounts for iron toxicity in these cells, which are prone, per se, to apoptosis, resulting in further progenitor loss and defective terminal erythroid maturation.[32-34]

Iron also exerts a detrimental action on the HSPC compartment in the MDS marrow. Iron overload in the MDS FPN^{C326S} mouse model promotes expansion of the LIP in HSPCs, which leads to higher cellular ROS production and increased cell susceptibility to apoptosis. Consistently, previous studies showed that iron overload impairs HSPC frequency and clonogenic capacity through ROS elevation. This mechanism likely plays a role in the exhaustion of the normal HSPC pool in MDS and may be prevented by limiting ROS formation through antioxidants or chelators.[11,13] Interestingly, LIP size is tightly regulated in HSPCs and linked to their quiescent and metabolically less-active functional state, preserving stemness and supporting stem cell regenerative capacity.[35,36] During aging, HSPCs undergo LIP expansion, which impairs HSPC self-renewal and attenuates iron-dependent cell fate control through metabolic alterations.[35] Thus, an altered LIP may play a role in the selection of preleukemic HSPC clones and increase the risk of MDS-to-AML transformation. Our data suggest that HSPCs in MDS mice look like "aged HSPCs," characterized by expanded LIP and loss of quiescence. This eventually results in primitive HSPC exhaustion, MP expansion, and disease progression. Our data also show that iron overload promotes DNA damage in MDS HSPCs, which is prevented by pharmacological iron restriction. Accordingly, iron excess has been implicated in oxidative DNA damage and telomere erosion in the BM of patients with MDS who have received transfusion,[37-40] being a potential source of genotoxic stress and an additional driver of genomic instability. Iron chelation improves the clonogenic capacity and limits DNA damage in ex vivo HSPCs from patients with MDS.[37] Overall, these observations suggest that, in MDS, iron contributes, on the 1 hand, to the exhaustion of normal HSPCs, and, on the other, to the clonality and myeloid skewing of a subset of mutated HSPCs through its genotoxic action. Indeed, mutated ring sideroblasts when exposed to excess ferrous iron upregulate antiapoptotic genes, indicating that mutated precursors have the ability to escape iron toxicity and avoid cell death by activating antiapoptotic programs, whereas normal HSPCs are progressively lost because of sensitivity to iron-induced cell death.[41] Thus, iron, despite lacking leukemic transformation ability, likely represents a cell-extrinsic advantage that directs clonal evolution and accelerates leukemic progression through oxidative and genotoxic stress in HSPCs.[1] This is supported by clinical data showing that iron-loaded patients with MDS present higher intracellular iron and ROS formations in BM CD34$^+$ cells, with ROS levels correlating with the percentage of BM blasts.[42,43] Indeed, AML transformation is more frequent in patients with iron overload compared with those without, strengthening the causal relation between increased body iron status and leukemic evolution.[42]

Our data show that iron exacerbates myeloid skewing in MDS by promoting MP expansion and myeloblast accumulation in

Figure 6. Combination therapy with VIT and luspatercept (LSPT) is superior compared with single therapies in the improvement of MDS pathophysiology. (A) Hb (i), RBC (ii), HCT (iii), MCV (iv), MCH (v); (B) Representative dot plots (i) and percentage of BM proerythroblasts, basophilic, polychromatic, and orthochromatic erythroblasts, reticulocytes, and erythrocytes (ii); Representative dot plots of immature CD71$^{high/int}$ and mature CD71^{low} Ter119$^+$ cells (iii); Representative dot plots of ROS$^+$ BM Ter119$^+$ cells (iv); percentage of annexin V$^+$ BM Ter119$^+$ cells (v) and representative dot plots (vi). (C) Percentage of BM LSK HSPCs (i) and LK MPs (ii) with representative dot plots (iii). (D) Flow cytometry analysis of apoptosis in BM LSK cells, expressed as MFI fold change (i) and percentage positive cells (ii). (E) Percentage of BM CD11b$^+$ (i-ii) and CD11b$^+$ Gr-1$^+$ myeloid cells (iii-iv), with representative dot plots (v), in wild-type and MDS mice receiving vehicle, VIT, or LSPT, alone or in combination. VIT was administered for 3 months, from 3 to 6 months of age; LSPT (mg/kg) was administered twice a week from 2 months, from 4 to 6 months of age. Data shown are averages of at least 3 independent experiments. Values represent mean ± SEM. Statistical analysis was performed by comparing ≥3 groups with 1-way ANOVA followed by Bonferroni posttest. *P < .05; **P < .01; ***P < .001; ****P < .0001.

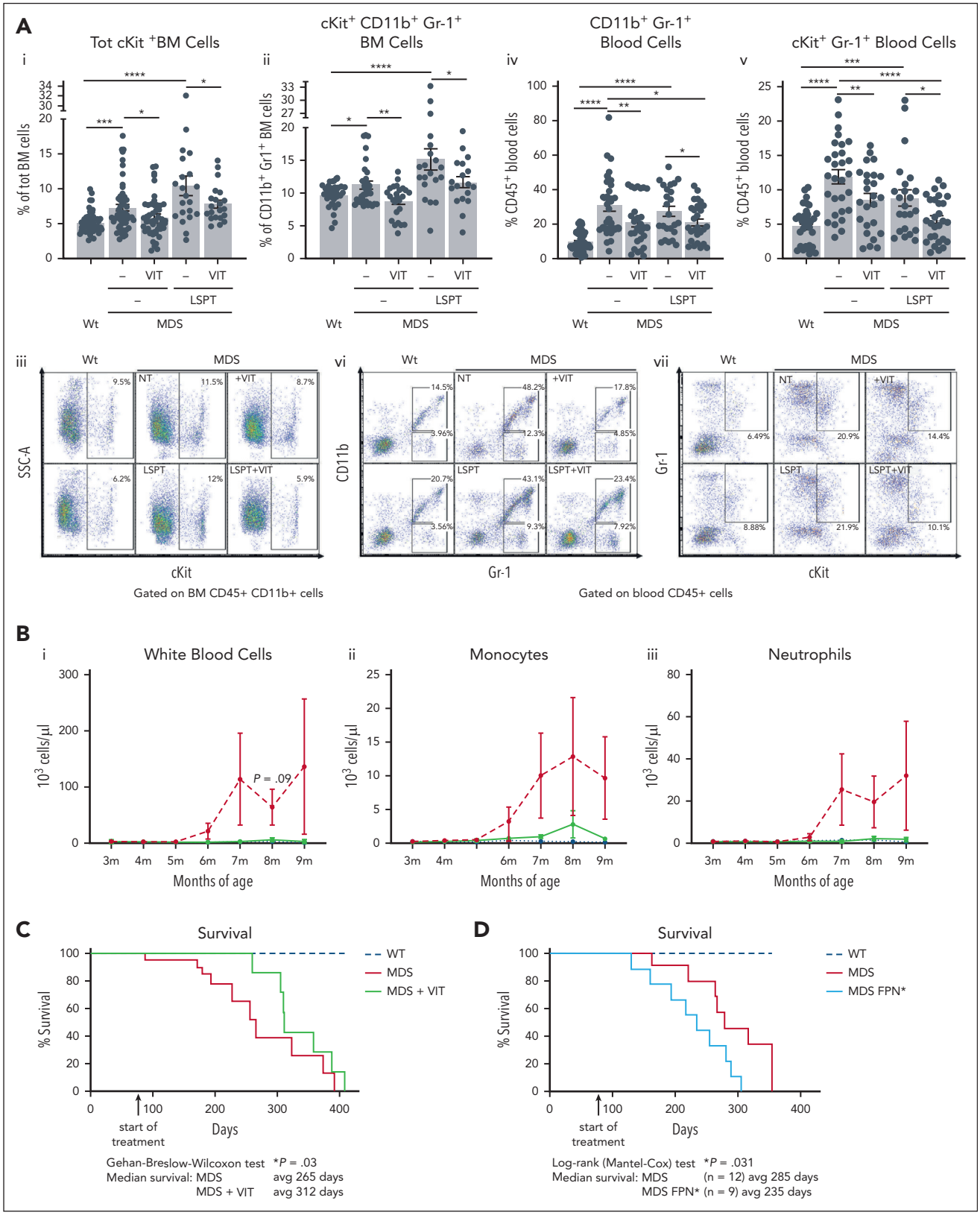

Figure 7. Iron restriction provides survival benefit in MDS. (A) Immature cKit$^+$ cells as percentage of total BM cells (i), CD11b$^+$ Gr-1$^+$ myeloid cells (ii), CD11b$^+$ Gr-1$^+$ cells (iii), and cKit$^+$ CD11b$^+$ Gr-1$^+$ cells (iv) as percentage of blood CD45$^+$ cells in wild-type mice and in MDS mice receiving vehicle, VIT or LSPT, alone or in combination. Representative dot plots are shown (iii,vi-vii). (B) Time-course of peripheral blood WBC (i), monocytes (ii), and neutrophils (iii) in wild-type and MDS mice receiving vehicle or VIT from 3 months of age onward. Data shown are average of 7 (Ai), 4 (Ai), and 10 (Aiii,iv) independent experiments. Values represent mean ± SEM. Statistical analysis was performed by comparing ≥3 groups with 1-way ANOVA followed by Bonferroni posttest. (C-D) Kaplan-Meier curve showing percentage survival of wild-type and MDS mice receiving vehicle or VIT for 3 months, and wild-type, MDS, and MDS FPN* mice. Statistical analysis was performed comparing the survival curves by log-rank Mantel-Cox test and Gehan-Breslow-Wilcoxon test. *P < .05; **P < .01; ***P < .001; ****P < .0001.

the BM and peripheral blood. Previous work suggests that iron overload in mice is sufficient to increase the number of BM MPs in absence of myelodysplasia.[44] Which mechanisms underlie iron-driven myeloid skewing in MDS remain to be clarified. This study shows that iron, by modulating myeloid bias, acts as disease modifier and may control leukemic evolution in MDS. In line with these findings, transfusional iron overload was shown to affect morbidity and mortality of lower-risk patients with MDS. Higher transferrin saturation (>80%) and ferritin (>800 μg/L), as well as hepatic iron overload are associated with inferior 5-year overall, progression-free, and leukemia-free survival.[45,46] In addition, transfusion dose density is inversely associated with progression-free survival, with a negative effect already observed at the relatively low transfusion densities of 3 units per 16 weeks.[47] However, a biphasic dose-response relationship has been shown to exist between AML rate and iron burden in preclinical studies: although iron elevation promotes leukemogenesis by inducing oxidative stress, mutagenesis, and myeloid expansion, supporting our observations, further iron elevation beyond a certain threshold rather decreases AML risk by increasing AML cell death,[48] adding complexity to the role of iron in MDS-to-AML evolution. This double dose-dependent behavior of iron likely explains the controversial observations obtained in clinical MDS real-life studies.[49]

In agreement with preclinical studies,[33] significant hematological improvement and ameliorated overall survival rate has been observed in lower-risk patients with MDS who received transfusions and chelation therapy, with 40% reaching an erythroid response.[34,50] In addition, event-free survival was longer in iron-chelated vs nonchelated patients with MDS.[51-53] However, the safety risks associated with the use of iron chelators call for the development of iron restriction drugs with fewer side effects, increased tolerability and better compliance. Our preclinical studies show that iron restriction obtained by reducing iron absorption and recycling via an oral FPN inhibitor improves anemia and provides survival benefit in MDS, offering an effective treatment with a safe profile. The observation that iron excess shortens the survival of both steady-state and iron-loaded MDS mice indicates that elevated iron promotes disease progression in both transfusion-dependent and -independent MDS. This is in agreement with observations showing that detectable labile plasma iron, the reactive NTBI fraction, is associated with inferior overall and progression-free survival in low-risk patients with MDS irrespective of transfusion status.[54,55]

MDS mice benefit from iron restriction by VIT early on, suggesting that iron-dependent mechanisms have an early relevance in MDS progression, whereas iron-independent mechanisms contribute to disease evolution at later stages. Interestingly, modest elevations of ferritin levels at diagnosis are independently associated with overall survival in patients with MDS, which might reflect an early role for iron in influencing prognosis in patients with MDS.[56] Overall, this highlights the importance of preemptive iron-reducing interventions, early use of chelation therapies or iron restriction approaches, and eventually more restrictive transfusion strategies in low-risk patients with MDS for the prevention of iron toxicities. Keeping with this concept, currently, trials are being designed with the aim of testing early application of iron chelation in low-risk patients with MDS who still show relatively modest iron loading.[57] Finally, our data emphasize how early treatment may prevent unnecessary morbidity in non–transfusion-dependent low-risk patients with MDS with symptomatic anemia, in whom disease burden is often insufficiently recognized, leading to delayed treatment.[58] MDS subtypes expected to benefit the most from inhibiting iron absorption are those with the lowest hepcidin levels and iron overload, which include MDS with ring sideroblasts and SF3B1 mutations,[59] and, to some extent, with deletion-5q, Tet2, and Asxl1 mutations.[7]

Ultimately, our work shows that the combination therapy with VIT and luspatercept has superior effects in erythropoiesis improvement compared with single treatments. This is in agreement with observations in β-thalassemia preclinical studies in which combined therapies with luspatercept and other iron restrictive approaches have been tested.[60,61] Iron restriction by VIT enhances the erythroid maturation action of luspatercept, likely by improving iron use in erythroid cells and ameliorating their survival. However, luspatercept does not have myeloid-modifying effect and does not reduce allelic burden.[62] Its administration together with VIT significantly improves myeloid expansion, showing disease-modifying activity with overall superior effects compared with single treatments. We believe that the combination of iron restriction strategies with erythroid maturating agents would provide a more efficient therapeutic option for MDS by improving anemia, reducing iron overload and limiting myeloid bias.

Despite being the best characterized murine model of MDS, reflecting most features of the human disease, the MDS mouse used for these studies has the limitation to model MDS associated with a quite rare human mutation, the NUP98-HOXD13 translocation.[22,23] Thus, it would be of interest to confirm our findings in other MDS mouse models with different and more common mutations (eg, Sf3b1, Tet2, and Dnmt3a). Future plans in the current MDS model include the evaluation of iron restriction vs overload on organ function and infection susceptibility, and the assessment of the combined effect of VIT with erythroid stimulating agents (eg, epoetin α), which act at different stages of erythroid maturation compared with luspatercept, as well as with RBC transfusions, in presence or absence of chelation therapy.

Overall, taking advantage of models of "pathophysiologic" iron overload and "pharmacological" iron restriction in MDS animals, these studies provide novel mechanistic insight on the role of iron in MDS as well as a preclinical proof-of-concept for an early application of therapeutic strategies to prevent NTBI formation and iron overload and, thus, delay leukemic transformation in patients with MDS.

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Authorship

Contribution: A.A. and S.Z.V. performed the in vivo experiments and measurements; R.S. and S.P. generated and characterized MDS

FPN^{C326S} mice; S.N. and M.A. performed histologic analysis and biochemical assays; F.D. and V.M. designed and supervised the study and contributed to manuscript preparation; and F.V. designed and supervised the study, conceived the experiments, analyzed data, wrote the manuscript, and prepared the figures.

Conflict-of-interest disclosure: F.D. and V.M. are employed at CSL Vifor. F.V. is a consultant for RallyBio and Pharmacosmos; and receives research fundings from CSL Vifor, which are relevant for the current studies, and Silence Therapeutics and PharmaNutra, which are not relevant to the current project. The remaining authors declare no competing financial interest.

ORCID profiles: V.M., 0000-0002-2072-8942; F.V., 0000-0003-1631-8286.

Correspondence: Francesca Vinchi, Iron Research Laboratory, Lindsley F. Kimball Research Institute, New York Blood Center, 310 E 67th St, New York, NY 10065; email: fvinchi@nybc.org.

Footnotes

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*A.A. and S.Z.V. contributed equally to this study.

†R.S. and S.P. contributed equally to this study.

Original data are available on request from the corresponding author, Francesca Vinchi (fvinchi@nybc.org).

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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