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The role of iron in mediating testosterone's effects on erythropoiesis in mice

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Abstract

Testosterone stimulates iron-dependent erythropoiesis and suppresses hepcidin. To clarify the role of iron in mediating testosterone's effects on erythropoiesis, we induced iron deficiency in mice by feeding low iron diet. Iron-replete and iron-deficient mice were treated weekly with testosterone propionate or vehicle for 3 weeks. Testosterone treatment increased red cell count in iron-replete mice, but, surprisingly, testosterone reduced red cell count in iron-deficient mice. Splenic stress erythropoiesis was stimulated in iron-deficient mice relative to iron-replete mice, and further increased by testosterone treatment, as indicated by the increase in red pulp area, the number of nucleated erythroblasts, and expression levels of TfR1, GATA1, and other erythroid genes. Testosterone treatment of iron-deficient mice increased the ratio of early-to-late erythroblasts in the spleen and bone marrow, and serum LDH level, consistent with ineffective erythropoiesis. In iron-deficient mice, erythropoietin levels were higher but erythropoietin-regulated genes were generally downregulated relative to iron-replete mice, suggesting erythropoietin resistance. Conclusion: Testosterone treatment stimulates splenic stress erythropoiesis in iron-replete as well as iron-deficient mice. However, testosterone worsens anemia in iron-deficient mice because of ineffective erythropoiesis possibly due to erythropoietin resistance associated with iron deficiency. Iron plays an important role in mediating testosterone's effects on erythropoiesis.

KEYWORDS

androgens, erythropoietin resistance, ineffective erythropoiesis, iron, iron deficiency

Abbreviations: ANOVA, analysis of variance; BMP, bone morphometric protein; CC3, cleaved caspase 3; CHr, reticulocyte hemoglobin concentration; DNA, deoxyribonucleic acid; ELISA, enzyme linked immunosorbent assay; EpoR, erythropoietin receptor; FOG-1, friend of GATA-1; GATA-1, GATA-binding factor 1, also known as erythroid differentiation factor; GDF11, growth and differentiation factor 11; H&E, hematoxylin and eosin; Hprt, hypoxanthine phosphoribosyltransferase; KLF1, an erythroid transcription factor, formerly known as EKLF; LDH, lactate dehydrogenase; MCV, mean corpuscular volume; mRNA, messenger ribonucleic acid; NFE2, Nuclear Factor, Erythroid 2; PBS, phosphate buffered saline; PCR, polymerase chain reaction; proE, proerythroblasts; qPCR, quantitative polymerase chain reaction; RBC, red blood cell; RDW, red blood cell distribution width; RNA, ribonucleic acid; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; TAL-1, T cell acute leukemia-1 factor; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

1 | INTRODUCTION

Testosterone increases hemoglobin, hematocrit, and circulating red cell counts in a dose-dependent manner.[1-6] Testosterone corrects unexplained anemia of the elderly, anemia of inflammation, and anemia of chronic kidney disease.[1,4,7-11] However, the mechanisms by which testosterone stimulates erythropoiesis remain incompletely understood.[3,12-16]

Several hypothesis have been proposed to explain the effects of testosterone on erythropoiesis, including stimulation of erythropoietin production; direct effects on erythroid progenitor cells; increased progenitor sensitivity to erythropoietin,[17,18] as well as increased iron availability.[19,20] We have shown that testosterone stimulates iron-dependent erythropoiesis in mice and in humans.[8-11] We further demonstrated that testosterone suppresses hepcidin transcription by disrupting BMP/Smad signaling,[21,22] and increases iron availability and iron incorporation into the erythroid cells.[22] However, additional experiments revealed that testosterone administration significantly increased hemoglobin and red cell counts even in mice with genetic disruption of the hepcidin gene.[23] Furthermore, a randomized trial of testosterone in older men with testosterone deficiency reported that testosterone treatment corrected unexplained anemia of the elderly as well as anemia of known causes including iron deficiency,[24] raising questions about the role of iron and hepcidin in mediating testosterone's effects on erythropoiesis.

The objective of the present studies was to clarify the role of iron in mediating the effects of testosterone on erythropoiesis. We generated a mouse model of iron deficiency by feeding wild-type young female mice a very-low iron diet for an extended period. The iron-deficient mice were then randomly assigned to receive either testosterone propionate in oil or oil alone. We surmised that if testosterone stimulates erythropoiesis primarily by stimulating iron-dependent erythropoiesis, its effects would be attenuated or abrogated in iron-deficient mice. The findings of these studies offer important insights into the role of iron in the mechanisms by which testosterone stimulates erythropoiesis and the role of iron in erythropoiesis.

2 | METHODS

2.1 | Animals

The animal experiments were performed in accordance with the National Institutes of Health Animal Care Guidelines and the research protocol was approved by the Animal Care and Use Committee at Brigham and Women's Hospital (protocol #:2016N000377). Female C57/BL6J mice (Jackson Laboratory, Bar Harbor, ME) were fed either regular diet with normal iron content (250 ppm iron, TD.110846, Harlan-Teklad, Madison, WI) or iron-deficient diet (3 ppm iron, TD.09127). The female mice were selected for these studies because we found that female mice exhibit a more consistent and slightly greater increase in hemoglobin in response to testosterone treatment than male mice. After 3 months, the mean hematocrits in the iron-restricted group gradually decreased from 0.50% (range: 0.47%-0.52%) to 0.30% (range: 0.28%-0.43%). Two weeks later, after anemia was stably established, mice on iron-restricted diet as well as those on regular diet were randomly allocated to receive either subcutaneous injections of testosterone propionate (50 mg/kg, weekly) or an equal volume of vehicle (oil) (100 μL) weekly for 3 weeks. A 3-week intervention duration was based on previous experiments in which significant increases in hemoglobin and hematocrit were observed in mice after 2 weeks of testosterone treatment. All injections were performed under mild isoflurane anesthesia.

2.2 | Blood analysis

Ten μl blood was acquired from the saphenous vein without anesthesia at baseline and weekly after the first injection. Hematocrits were measured after centrifugation using a StatSpin device. Complete blood counts were measured at the end of the intervention period in EDTA-anticoagulated blood samples obtained by cardiac puncture using Bayer Advia 1200 System with mouse-specific software.

2.3 | Serum and tissue nonheme iron analysis

Serum nonheme iron and UIBC (the iron binding sites on transferrin that are not occupied by iron) were measured using a commercial assay kit (#17504, Pointed Scientific). To measure liver nonheme iron, 60-75 mg of liver tissue was digested in 1 mL of 3 M hydrochloric acid and 0.61 M trichloroacetic acid at 60°C for 48 hours. After addition of a chromogen mix containing 0.31 mM of bathophenanthroline sulfonate and 23.83 mM of thioglycolic acid in 5 volumes of saturated sodium acetate, iron concentration was measured by optical density at 535 nm in reference to a calibration curve.[22]

2.4 | Other serum analysis

Serum ferritin was measured using ELISA (Abcam ab157713). Serum lactate dehydrogenase (LDH) was measured using assay reagents from Sigma-Aldrich (MAK066-1KT). Serum erythropoietin concentration was measured using an ELISA kit from R&D System (MEP00B).

2.5 | Reverse-transcription quantitative polymerase chain reaction

Small fragments of kidney and spleen were homogenized in TRIzol. Total RNA was isolated using Direct-zol RNA Miniprep kit. Genomic DNA was removed by in-column digestion with DNAse I (R2051, Zymo Research, Irvine, CA). Reverse transcription was performed using a first strand cDNA synthesis kit (E6300L, New England Biolab, Ipswich, MA). Real-time PCR was performed on an ABI7500 real-time PCR system (#4406985, Thermo Fisher) using a standard protocol based on SYBR Green Mastermix (A25743, Thermo Fisher). Intron-spanning primers were designed using Pick Primer service provided by National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). The primer sequences are provided in Supporting Information Table S1. All qPCR results were referenced to the expression level of house-keeping gene hypoxanthine phosphoribosyltransferase (*hprt)* or other reference genes, as specified.

2.6 | Histology and immunohistochemistry

Upon euthanasia, the spleen was dissected, weighed, and cut into sections: the proximal third was used to isolate single cells (see below), the distal third was fixed in PBS-buffered formalin (10%), and the middle third was frozen for RNA analysis. Femur was dissected, cleaned off the attached muscle, and immersed in PBS-buffered formalin after removal of the epiphyses at both ends. After 96 hours, the bone was decalcified by immersion in 5% of formic acid for 22 hours and rinsed with water. Both spleen and bone samples were submitted for processing and staining (H&E and Iron) by the Rodent Histopathology Core, Harvard Medical School. Immunohistochemistry for transferrin receptor 1 was performed by the Pathology Core at Boston Children's Hospital. Additional immunostaining was performed for GATA-1 (rabbit monoclonal #3535S, Cell Signaling), GDF11 (rabbit polyclonal #ab199139, Abcam), cleaved caspase 3 (rabbit monoclonal #9661S, Cell Signaling), and Ter119 (biotin-conjugated #116203, Biolegend), following standard protocols. Briefly, after dewax, antigen retrieval was performed by immersing the slides in rodent decloker (#RD913, Biocare Medical) heated to 94°C for 40 minutes in a decloking chamber. The slides were then treated with Bloxall (#SP-6000, Vectorlabs) for 10 minutes to block endogenous peroxidase, followed by blocking nonspecific antibody binding using Background Punisher (#BP974, Biocare Medical). Primary antibodies were diluted in Da Vinci Green Diluent (#PD900, Biocare) at a ratio of 1:200 to 1:1000, and applied to sections at 4°C overnight. After washing, Rabbit-on-Rodent HRP Polymer (#RMR622, Biocare) or Impress-AP-Rabbit IgG Polymer (#SMP-5401, Vectorlab) was applied at room temperature for 20 minutes. For Ter119 staining, additional blocking were performed using the Avidin/Biotin blocking kit (#004303, Life Technologies Frederick, MD) before applying the primary antibody, and biotinylated anti-Ter119 was detected using the VectorStain RTU Elite ABC HRP reagent (#PK-7100, Vectorlab). After washing, slides were developed with impact DAB (#SK-4105, Vectorlab) or Vector Blue (#SK-5300) accordingly.

Results of immunostaining were evaluated by blinded scoring of four randomly selected views in each slide using a 1 to 4 scale, with 4 being the highest staining intensity. For targets with more abundant expression, staining was quantified by densitometry using NIH image J.

2.7 | Flow cytometry

A section of the spleen was minced in ice-cold PBS containing 2% of fetal bovine serum. Single cells were prepared by passing the minced tissue through a 20G needle six times. After passing through a 70 μm strainer, red blood cells were lysed using RBC lysis buffer (#420301, Biolegend). Cells were double-stained with PE anti-mouse CD71 (#113807, Biolegend) and Pacific Blue anti-mouse Ter119 (#116231, Biolegend). Dead cells were excluded as those staining positive for 7AAD (#420403, Biolegend). The flow cytometry analyses were conducted using a MACSQuant VYB flow cytometer (Miltenyi Biotec, Somerville, MA) and analyzed using FlowJo software version 10.0 (FlowJo LLC, Ashland, Oregon).

2.8 | Statistical analyses

Statistical analyses were performed using Prism software (version 8.0.1; GraphPad Software, Inc San Diego, CA, USA). Most of the results are presented as dot plots with each dot representing an individual sample. Unpaired *t* tests for independent samples were performed for two-group comparisons. One-way analysis of variance (ANOVA) was performed to assess effect of treatment when there were more than two intervention arms. When overall ANOVA revealed a significant treatment effect, Tukey's honest significance test was used to assess the difference between individual groups. Type 1 error was set at 0.05 level.

3 | RESULTS

3.1 | Low iron diet results in systemic iron depletion and microcytic anemia

As expected, compared to mice fed normal iron diet, the mice fed low iron diet had 50% to 75% lower liver nonheme iron

stores ($P = .001$) and significantly lower circulating levels of ferritin ($P = .0001$) and iron ($P = .003$), and lower transferrin saturation ($P = .0001$) (Figure 1). Prussian Pearl staining revealed a marked reduction of splenic nonheme iron, with very little detectable blue pigment in the mice fed low iron diet, compared to those fed normal iron diet (Figure S1). Consistent with diminished body iron stores, hemoglobin ($P < .0001$; Figure 2A), hematocrit ($P < .0001$) (Figure 2B), MCV ($P < .0001$; Figure 2C), and reticulocyte hemoglobin concentration (CHr) ($P = .003$; Figure 2D) were substantially lower, and RDW ($P = .001$; Figure 2E) was significantly higher in the mice fed low iron diet than in mice fed normal chow.[25] Red blood cell counts did not differ significantly between the iron-replete and iron-deficient groups (Figure 2F), suggesting that the body responded to iron deficiency by producing less hemoglobin per cell and smaller cells, as previously reported.[26]

3.2 | Testosterone administration to mice fed low iron diet worsens anemia

As expected, testosterone administration to mice fed normal iron diet increased hemoglobin ($P = .009$), hematocrit ($P = .008$), and red cell counts ($P = .01$; Figure 2). In contrast, testosterone treatment of mice fed low iron diet was associated with significantly lower hemoglobin (($P = .007$), hematocrit ($P < .001$)), and red cell counts (PP = 0.01), and higher RDW ($P = .009$) than mice fed low iron diet and treated with vehicle (Figure 2).

Testosterone treatment of mice fed regular iron diet increased serum iron and transferrin saturation and reduced liver iron and serum ferritin levels as compared to the vehicle-treated group (Figure 1), consistent with our previous observations that testosterone increases iron availability and utilization.[22] However, testosterone treatment of iron-deficient mice did not significantly affect serum iron levels, transferrin saturation, or liver iron stores, and increased serum ferritin (Figure 1).

3.3 | The effect of testosterone on splenic stress erythropoiesis in iron-deficient mice

In rodents, circulating hematopoietic progenitors, originating in the bone marrow, home into the spleen where they expand and mature into erythrocytes to be released into the circulation. In response to stress, such as that induced by anemia, the spleen becomes the major site of stress erythropoiesis in mice.

The iron-deficient mice had a modest increase in spleen volume and spleen-to-body weight ratio (Figures S2A and S3A), compared to iron-replete mice, consistent with previous observations.[27] Testosterone treatment of iron-deficient mice was associated with a marked increase in spleen size and spleen weight-to-body weight ratio,

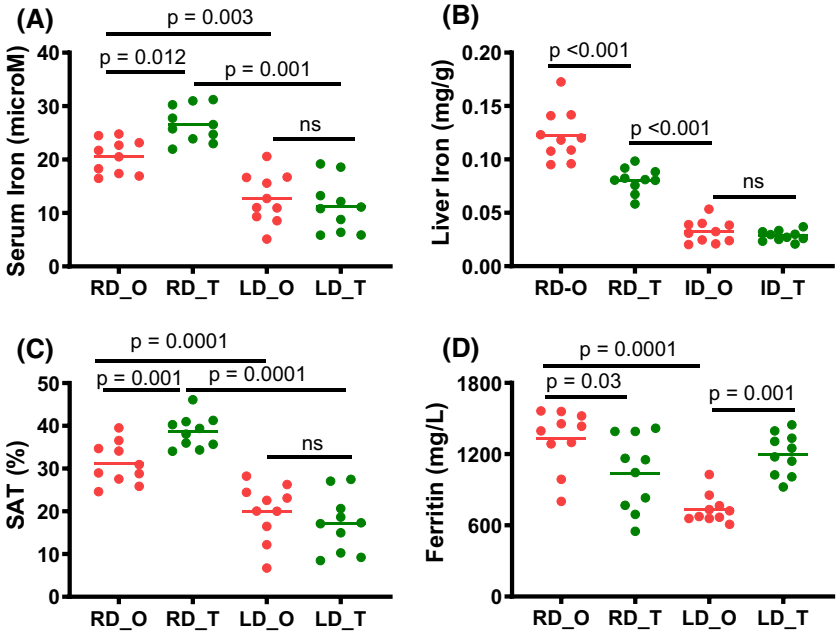

FIGURE 1 Effects of testosterone treatment in mice fed regular iron diet or low iron diet on (A) serum iron concentration, (B) liver iron concentration, (C) transferrin saturation (SAT), and (D) serum ferritin concentration. Each data point represents one animal; n = 10 in each group. Data were analyzed by one-way ANOVA followed with Tukey's test for between-group comparison. RD_O, mice fed regular iron diet, and treated with oil alone; RD_T, mice fed regular iron diet and treated with testosterone; LD_O, mice fed low iron diet and treated with oil alone; LD_T, mice fed low iron diet and treated with testosterone. *P* values for the indicated comparisons are shown. NS, not statistically significant by Tukey's test.

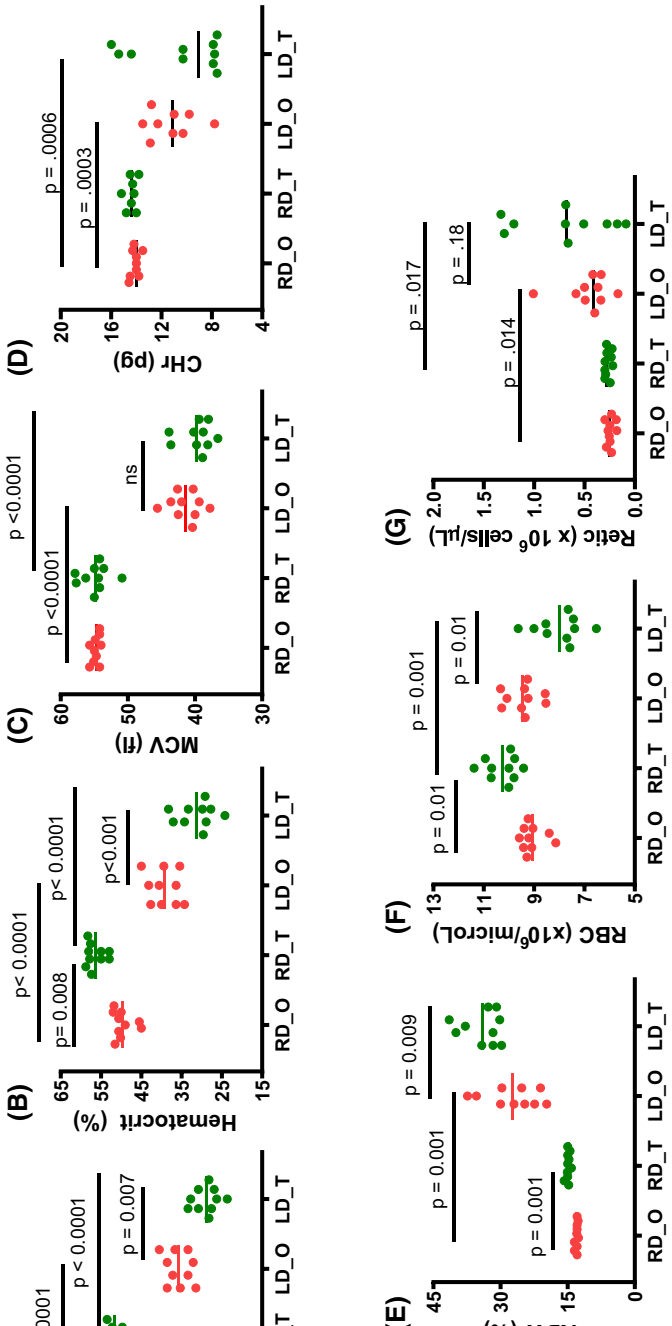

FIGURE 2 Effects of testosterone or oil treatment in mice fed regular iron diet or low iron diet on: (A) Hemoglobin, (B) hematocrit, (C) mean corpuscular volume, (D) reticulocyte hemoglobin, (E) red cell distribution width, (F) red blood cell count, and (G) reticulocyte count. Each data point represents one animal (n = 10). Data were analyzed by one-way ANOVA followed with Tukey's test for intergroup comparison. RD_O, mice fed regular iron diet, and treated with oil alone; RD_T, mice fed regular iron diet and treated with testosterone; LD_O, mice fed low iron diet and treated with oil alone; LD_T, mice fed low iron diet and treated with testosterone. NS, not statistically significant by Tukey's test

compared to iron-deficient mice treated with vehicle alone as well as iron-replete mice treated with oil or testosterone (Figures S2A and S3A).

Hematoxylin and eosin (H&E) staining of the spleen revealed substantial changes in the relative proportion of red to white pulps in response to induction of iron deficiency and testosterone treatment. Compared to vehicle treatment, testosterone treatment of iron-replete mice was associated with a significantly larger red pulp area ($P < .0001$; Figures 3B and S2B upper panels). Testosterone-treated iron-deficient mice had significantly greater red pulp area and a higher number of nucleated erythroblasts in the red pulp than vehicle-treated iron-deficient mice (Figures 3 and S2B). In higher magnification views, the substantial increase in the density of nucleated erythroblasts is qualitatively apparent in the red pulp of iron-deficient mice treated with testosterone compared to all other groups (Figure S2C).

Transferrin receptor 1 (TfR1) is a membrane iron transporter that facilitates the transport of transferrin-bound iron from circulation into differentiating erythroblasts for hemoglobin synthesis. Immunostaining revealed that expression of TfR1 protein in the red pulp was significantly higher in iron-deficient mice than in iron-replete mice ($P < .0001$); testosterone treatment of iron-deficient mice was associated with even higher TfR1 expression than vehicle treatment ($P = .009$; Figures 3C and S2D,E). Similarly, TfR1 mRNA expression levels were significantly higher in iron-deficient mice compared to mice fed normal iron diet ($P = .002$); testosterone treatment of iron-deficient mice further increased TfR1 mRNA expression ($P = .036$; Figure 3D). In line with these findings, the nuclear expression of the erythroid transcription factor GATA binding protein 1 (GATA1) ($P = .03$; Figure 3E) as well as GATA1 mRNA ($P = .006$; Figure 3F) were significantly higher in the red pulp of the iron-deficient mice, compared to iron-replete mice. Testosterone treatment of iron-deficient mice was associated with significantly higher GATA1 protein ($P = .01$) as well as GATA1 mRNA ($P = .029$) expression level than vehicle treatment (Figures 3E,F and S2F,G).

The mRNA expression levels of several other key transcription factors and cofactors of erythropoiesis, including FOG, KLF1, NFE2, and TAL1, were also higher in the iron-deficient mice compared to iron-replete mice; testosterone treatment of iron-deficient mice further increased the mRNA expression levels of these transcription factors and cofactors of erythropoiesis (Figure S3). Thus, splenic stress erythropoiesis was greatly enhanced in iron-deficient

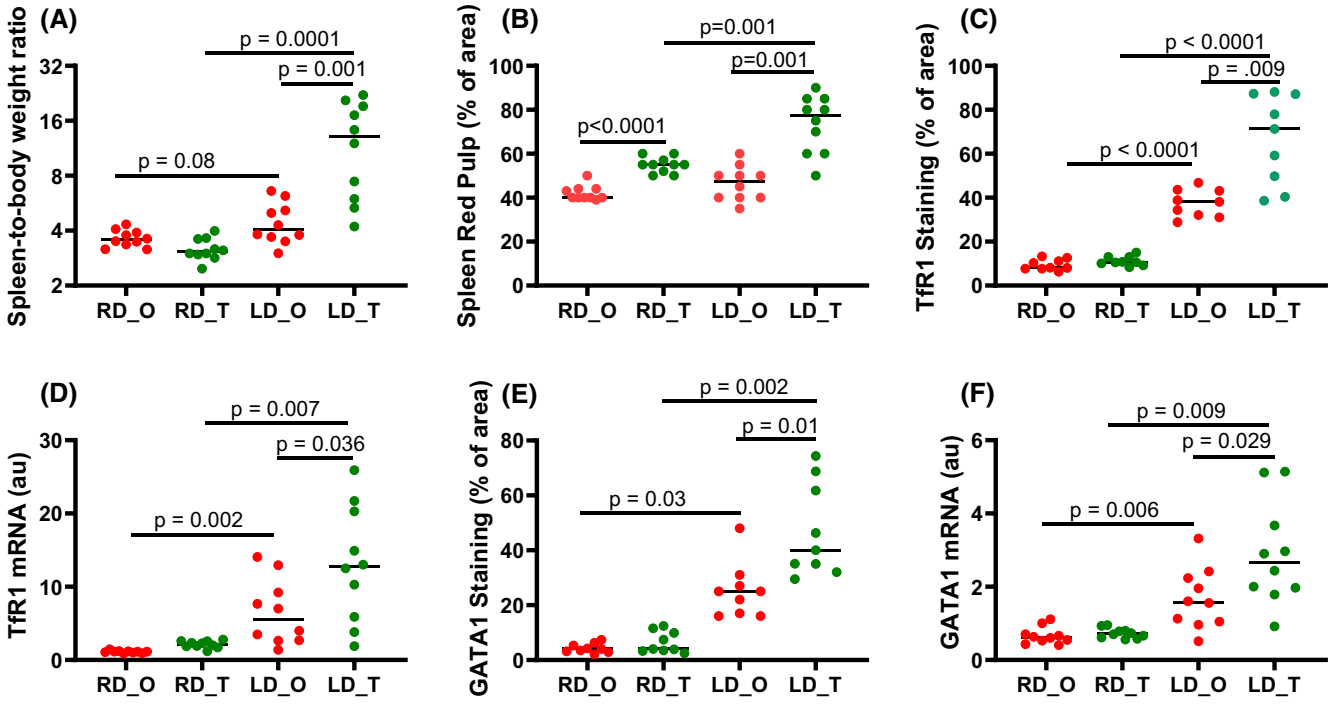

FIGURE 3 Effects of testosterone or oil treatment in mice fed regular iron diet or low iron diet on: (A) spleen-to-body weight, (B) spleen red pulp area expressed as a percent of total spleen cross-sectional area, (C) immunohistochemical staining for transferrin receptor 1, (D) splenic mRNA expression of transferrin receptor 1, (E) splenic immune staining for GATA1, (F) splenic mRNA expression of GATA1. Each data point represents one animal; n = 10 per treatment group. Data were analyzed by one-way ANOVA followed with Tukey's test for post hoc comparison. RD_O, mice fed regular iron diet, and treated with oil alone; RD_T, mice fed regular iron diet and treated with testosterone; LD_O, mice fed low iron diet and treated with oil alone; LD_T, mice fed low iron diet and treated with testosterone. NS, not statistically significant by Tukey's test; Epo, erythropoietin

mice and further enhanced after testosterone treatment of iron-deficient mice, as indicated by the substantial increase in the red pulp area, the number of nucleated erythroblasts, and the increased expression of TfR1 and GATA1 protein and mRNA levels and other erythroid factors (Figures 3, S2, and S3).

3.4 | Testosterone treatment of iron-deficient mice is associated with substantially increased ratio of early to late erythroblasts

As shown above, in spite of the stimulation of erythropoietic activity, testosterone treatment worsened anemia in iron-deficient mice. Therefore, we considered the possibility that testosterone treatment of iron-deficient mice was associated with ineffective erythropoiesis.

We used flow cytometry for cell surface expression of CD71 (TfR1) and Ter119 to categorize the splenic erythroblasts as proerythroblasts (proE; CD71+, Ter119-), basophilic and polychromatophilic erythroblasts (CD71+, Ter119+), and orthochromatic erythroblasts (CD71-, Ter119+).[28] As shown in Figure 4A, in mice fed normal iron diet, the erythroblasts contributed only a small fraction (~10%) of the total spleen cells, as previously reported,[29] and the erythroblast pool consisted predominantly of orthochromatic erythroblasts. Testosterone treatment of iron-replete mice modestly increased the proportion of splenic erythroblasts, primarily by an increase in the basophilic-polychromatophilic erythroblasts, as we have reported previously.[30] Experimentally induced iron deficiency was associated with ~300% increase in splenic erythroblasts, largely by increasing the basophilic-polychromatophilic erythroblasts (nearly 900%) and proerythroblasts (nearly 600%); iron-deficient mice had substantially fewer orthochromatic erythroblasts (~80% decrease), in comparison to the iron-replete mice (Figure 4). Testosterone treatment of iron-deficient mice further increased the proportion of erythroblasts largely by increasing the absolute number and proportion of proerythroblasts and basophilic-polychromatophilic erythroblasts, without changing the number of orthochromatic erythroblasts, compared to iron-deficient mice treated with vehicle. Because the spleen volume was markedly increased by testosterone treatment in iron-deficient mice (Figures 3A and S2A), the number of orthochromatic erythroblasts in the whole spleen was higher in the testosterone-treated iron-deficient mice than in the vehicle-treated iron-deficient mice, but was still significantly lower than that of the iron-replete groups (Figure 4B, lower panel). Thus, testosterone treatment of iron-deficient mice was associated with substantial increase in the numbers of proerythroblasts and basophilic-polychromatophilic erythroblasts but did not improve their maturation into orthochromatic erythroblasts resulting in substantial increase in the ratio of early to late erythroblasts.

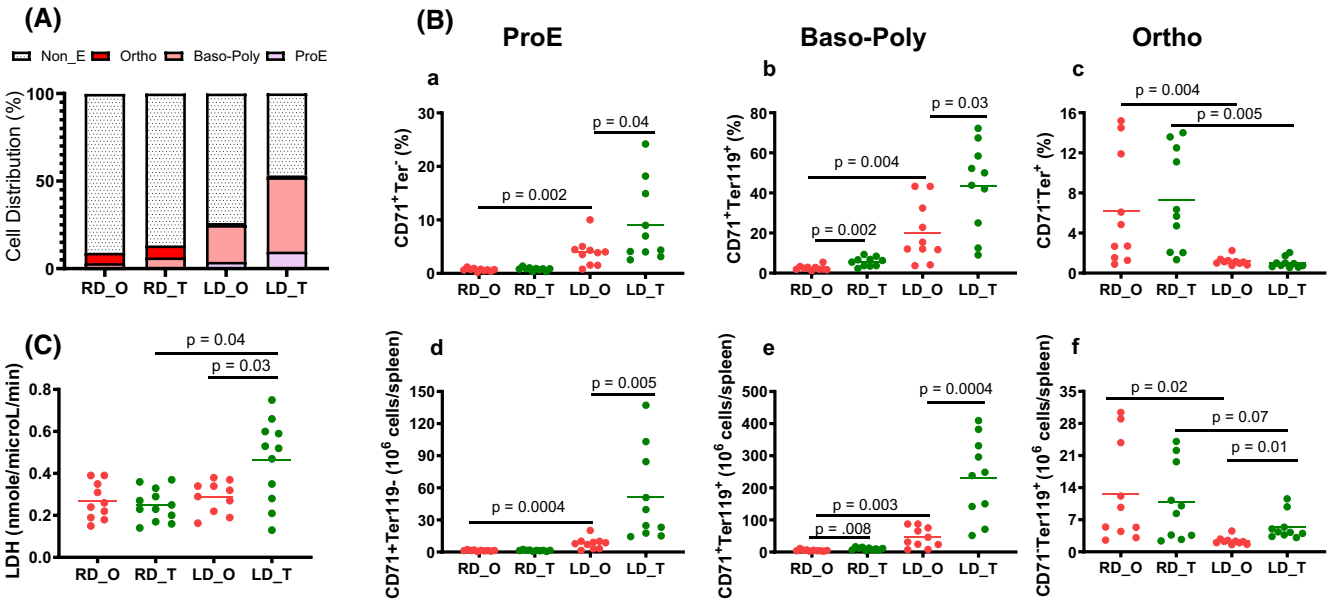

FIGURE 4 Effects of testosterone or oil treatment in mice fed regular iron diet or low iron diet on: (A) the distribution of non-erythroblasts (Non_E), proerythroblasts (ProE), baso-polychromatic erythroblasts (Baso-Poly), and orthochromatic erythroblasts (Ortho), (B) distribution of erythropoietic progenitors expressed as percentage of proerythroblasts (a: $CD71^{+}Ter119^{-}$), baso-polychromatic erythroblasts (b: $CD71^{+}Ter119^{+}$), and orthochromatic erythroblasts (c: $CD71^{-}Ter119^{+}$), and as the absolute number of cells at the corresponding stages in the spleen (d, e, f). (C) serum lactate dehydrogenase activity. Each data point represents one animal; n = 10. Data were analyzed by one-way ANOVA followed with Tukey's test for intergroup comparison

3.5 | Effects of testosterone on the expression levels of erythropoietin, erythropoietin receptor, transferrin receptor 2, and erythropoietin-regulated genes

Although kidney mass was significantly lower in iron-deficient mice compared to mice fed normal iron diet, testosterone treatment was associated with a significant increase in kidney mass in iron-replete as well as iron-deficient mice (Figure 5A) indicating that the iron-deficient mice were responsive to the known anabolic effect of testosterone on kidney mass. Renal erythropoietin mRNA expression levels and serum erythropoietin concentration were markedly higher in the iron-deficient mice, as compared to the iron-replete groups (Figure 5B,C).[26,31] Erythropoietin levels did not differ significantly between vehicle-treated vs testosterone-treated mice regardless of the iron status (Figure 5B,C).

The splenic mRNA expression of Erythropoietin Receptor (EpoR) and transferrin receptor 2 (TfR2) (Figure S3A, lower panel) did not differ across groups. However, because the proportion of erythroblasts per unit spleen mass was substantially higher in the iron-deficient compared to iron-replete mice, we normalized the expression level of these erythroid markers to the expression level of GATA1 as a marker of splenic erythroid progenitors (Figure 5D,E). When normalized to the corresponding GATA1 expression level, the mRNA expression levels of EpoR and TfR2, were significantly downregulated in both groups of iron-deficient mice (Figure 5D,E), compared to the iron-replete groups, consistent with previous studies showing that iron deficiency downregulates splenic expression of TfR2 in erythroblasts.[32]

TfR2 and EpoR are normally activated synchronously and coexpressed during terminal erythropoiesis from proerythroblasts to basophilic erythroblasts.[28] Unlike TfR1 that transports holotransferrin-bound iron into the cells through internalization, TfR2 binds holotransferrin but remains on the plasma membrane to help stabilize EpoR anchorage and to optimize erythropoietin signaling.[28] The knockdown of TfR2 delays terminal differentiation of human erythroid progenitors.[33] In iron deficiency, unbound TfR2 is targeted to lysosome degradation, resulting in a reduction of cell surface distribution of EpoR and an inhibition of erythroid differentiation.[34] Consistent with these earlier reports, our data show that iron deficiency downregulates TfR2 and EpoR that may contribute to inhibition of terminal erythroid differentiation.

We considered the possibility that the marked increase in the ratio of proerythroblasts plus basophilic-polychromatophilic erythroblasts to orthochromatic erythroblasts in testosterone-treated iron-deficient mice might be the result of ineffective erythropoiesis due to erythropoietin resistance. Indeed, we found that the expression levels of erythropoietin-regulated genes, such as HBB and cyclin D1, were lower in iron-deficient mice than in iron-replete mice, in spite of markedly elevated circulating erythropoietin levels

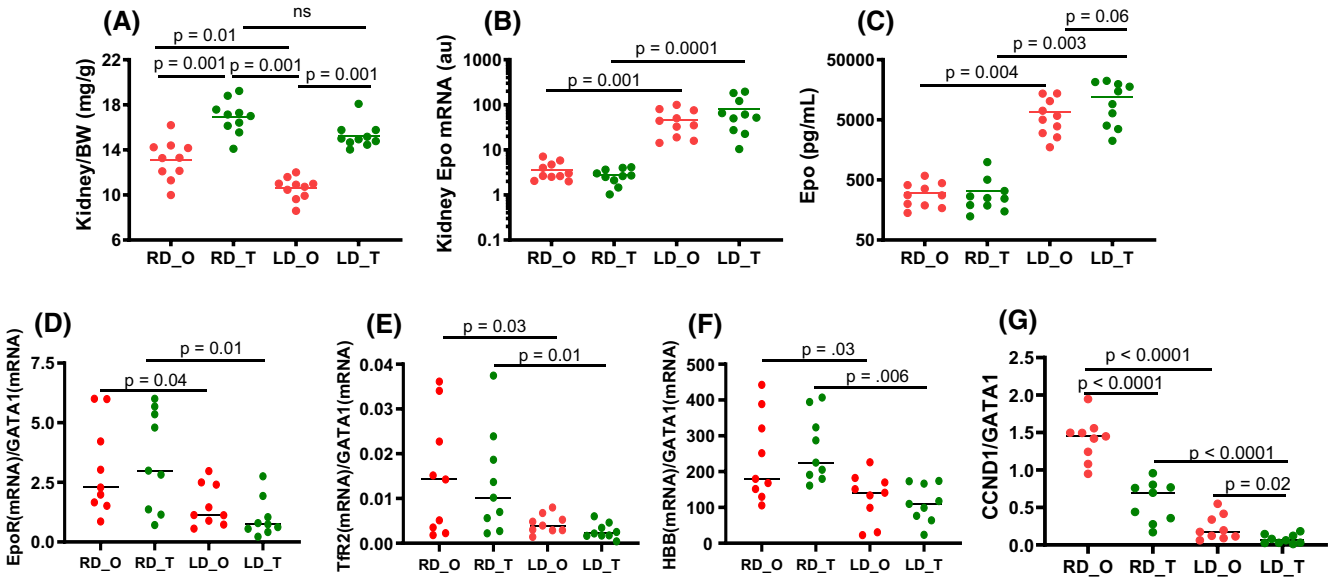

FIGURE 5 Effects of testosterone or oil treatment in mice fed regular iron diet or low iron diet on: (A) Kidney-to-body weight, (B) renal erythropoietin mRNA expression, (C) serum erythropoietin concentration. (D) ratio of the splenic mRNA expression levels of erythropoietin receptor (EpoR) to GATA1, (E) ratio of the splenic mRNA expression levels of transferrin receptor 2 (TfR2) to GATA1, (F) ratio of splenic mRNA expression levels of hemoglobin beta chain (HBB) to GATA1, (G) ratio of splenic mRNA expression levels of cyclin D1 to GATA1. Each data point represents one animal (n = 10). Data were analyzed by one-way ANOVA followed with Tukey's test for post hoc comparison. RD_O, mice fed regular iron diet, and treated with oil alone; RD_T, mice fed regular iron diet and treated with testosterone; LD_O, mice fed low iron diet and treated with oil alone; LD_T, mice fed low iron diet and treated with testosterone. NS, not statistically significant by Tukey's test; Epo, erythropoietin

(Figure 5), suggesting that iron deficiency was associated with some level of erythropoietin resistance. Testosterone treatment of iron-deficient mice was associated with higher serum LDH levels compared to vehicle-treated iron-deficient mice (Figure 4C), suggesting that testosterone treatment had worsened ineffective erythropoiesis in iron-deficient mice.

We further evaluated whether this was related to increased apoptosis as a result of diminished erythropoietin signaling. However, immunohistochemical staining revealed only a small number of cells positive for cleaved caspase 3 (CC3), a marker for apoptosis that typically accounts for only ~0.5%-1.0% of the cells in a normal murine spleen.[35] CC3+ staining did not differ among groups (Figure S3B). Thus, these results did not provide clear evidence of a detectable increase in splenic apoptosis.

3.6 | Testosterone treatment of iron-deficient mice enhances bone marrow erythropoiesis and increases the ratio of early erythroid progenitors and the late erythroblasts

In adult mammals, bone marrow is the primary source of erythroid progenitors under physiologic conditions, and spleen is the dominant site of stress erythropoiesis when mice become anemic.[36,37] Accordingly, in addition to characterizing the splenic erythropoiesis, the major site of stress erythropoiesis in anemic mice, we also evaluated how the bone marrow responded to iron deficiency and testosterone treatment.

The femur bone marrow was stained for GATA1 and Ter119 as markers of erythropoiesis. GATA-1 is the essential transcription factor which begins to be expressed early in hematopoiesis to regulate erythroid lineage commitment and differentiation. Expression of GATA1 begins to decline at the beginning of terminal erythropoiesis,[38,39] whereas Ter119 begins to be expressed at the beginning of terminal erythropoiesis and its expression is maintained throughout the life span of mature red blood cells.[40-42] As shown in Figure 6A, nuclear staining of GATA1 was detected in ~40% of the bone marrow cells of the iron-replete mice, as expected. Testosterone treatment of iron-deficient mice was associated with an increase in the proportion of GATA1-expressing cells (~60%) consistent with stimulation of erythropoietic progenitors (Figure 6A,C).

In iron-replete mice, cell surface staining for Ter119 was detected in ~40% of bone marrow cells (Figure 6B,D), and the proportions of cells staining positive for GATA1 and Ter119 were nearly equal (Figure 6E). However, in the testosterone-treated iron-deficient mice, the proportion of cells expressing GATA1 was increased (~60%) but only ~ 40% of the bone marrow cells stained positive for Ter119 (Figure 6D), resulting in an increased ratio of GATA1+ early erythroid progenitors to the Ter119+ late erythroblasts (Figure 6E).

To investigate whether the increased ratio of early to late erythroblasts in the bone marrow in testosterone-treated iron-deficient mice (LD_T group) could be explained by increased apoptosis, immunostaining for CC3 was performed. CC3 staining was increased in iron-deficient mice (Figure S4A) treated with vehicle and testosterone compared to the mice fed normal diet. The bone marrow immunohistochemical staining revealed increased GDF11 expression (which has been implicated in ineffective erythropoiesis in other conditions, such as thalassemia) in the iron-deficient mice in comparison to iron-replete mice (Figure S4B). However, the GDF11 expression did not differ between the iron-deficient mice treated with vehicle or testosterone.

4 | DISCUSSION

The findings of the present study offer insight into the role of iron in erythropoiesis and in mediating the mechanisms by which testosterone stimulates erythropoiesis. Our findings indicate that iron availability plays an essential role in mediating erythropoietic response to testosterone, and that testosterone treatment of iron-deficient mice worsens anemia. Testosterone administration of iron-deficient mice was associated with a marked stimulation of stress erythropoiesis in the spleen, as indicated by an increase in spleen size, red pulp area, and the number of erythroblasts in the spleen, as well as increased expression of GATA1, TfR1, and other markers of erythropoiesis. Multiple observations provide evidence that the anemia in iron-deficient mice was worsened by testosterone treatment because of ineffective erythropoiesis. First, there was substantial expansion of early erythroid progenitors in response to testosterone treatment of iron-deficient mice, as indicated by increased numbers of early erythroblasts as well as increased expression of GATA1, TfR1, and other erythroid genes. Second, there was a maturation block at the polychromatophilic stage, as indicated by a substantially increased ratio of proerythroblasts plus polychromatophilic erythroblasts to orthochromatic erythroblasts in iron-deficient mice treated with testosterone. Finally, there was a loss of erythroid precursors as indicated by fewer late erythroblasts, worsened anemia in spite of evidence of markedly stimulated stress erythropoiesis in the spleen, and increased LDH levels.

Because of the abundance of iron in the rodent chow, the induction of iron deficiency in laboratory mice takes a long period of feeding of low iron diet. However, multiple lines of evidence confirmed that feeding of low iron diet substantially depleted the body's iron stores in the experimental mice (liver nonheme iron stores, low serum ferritin, reduced serum iron

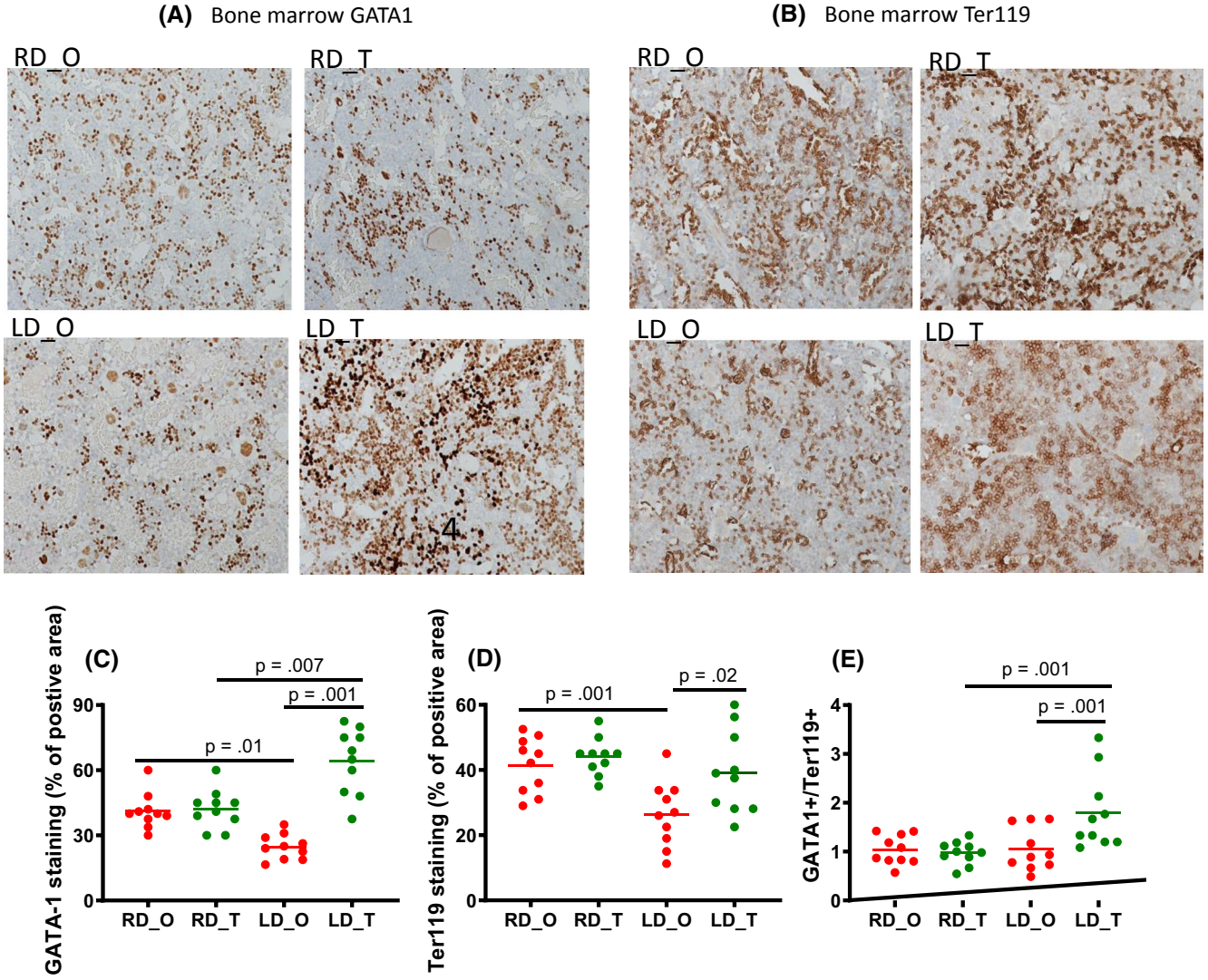

FIGURE 6 Effects of testosterone or oil treatment in mice fed regular iron diet or low iron diet on bone marrow expression of early (GATA1) and terminal (Ter119) markers of erythropoiesis. Representative microphotograph of immunohistochemical staining of bone marrow for GATA1 (A) and (B) for Ter119 (both 200 X). C and D, The results of the immunohistochemical staining of bone marrow quantified by Image J for GATA-1 (C) and Ter119 (D). Panel E shows the ratio between Ter119 and GATA-1 staining in the bone marrow. For C-E, n = 10 in each treatment group. Data were analyzed by one-way ANOVA followed with Tukey's test for intergroup comparison. RD_O, mice fed regular iron diet, and treated with oil alone; RD_T, mice fed regular iron diet and treated with testosterone; LD_O, mice fed low iron diet and treated with oil alone; LD_T, mice fed low iron diet and treated with testosterone. NS, not statistically significant by Tukey's test

and transferrin saturation, and marked reduction in stainable iron in the spleen) and induced a state of physiological iron deficiency (reduced hemoglobin, hematocrit, MCV, and reticulocyte hemoglobin levels). The mice fed low iron diet had a substantial increase in the red pulp area, the number of nucleated erythroblasts, and in the expression levels of GATA1 and TfR1 protein and mRNA expression levels, suggesting that iron deficiency was associated with an expansion of early erythropoietic progenitor pool and a stimulation rather than suppression of stress erythropoiesis in the spleen. The number of proerythroblasts and polychromatophilic erythroblasts in the spleen was increased markedly in iron-deficient mice compared to iron-replete mice. The increased ratio of proerythroblasts plus polychromatophilic erythroblasts to orthochromatic erythroblasts suggests a maturation block at the polychromatophilic erythroblast stage leading to ineffective erythropoiesis. Iron deficiency is known to induce a state of erythropoietin-resistance,[43] which contributes to the reduced survival of late erythroblasts. This proposal is supported by our findings that in spite of markedly elevated erythropoietin levels in iron-deficient mice, the expression of erythropoietin-regulated genes, such as HBB and cyclin D1, was suppressed significantly.

Our findings are consistent with a growing body of preclinical[21,22] as well as clinical studies[3,5,12,14,19,20] that suggest that testosterone administration increases hemoglobin and

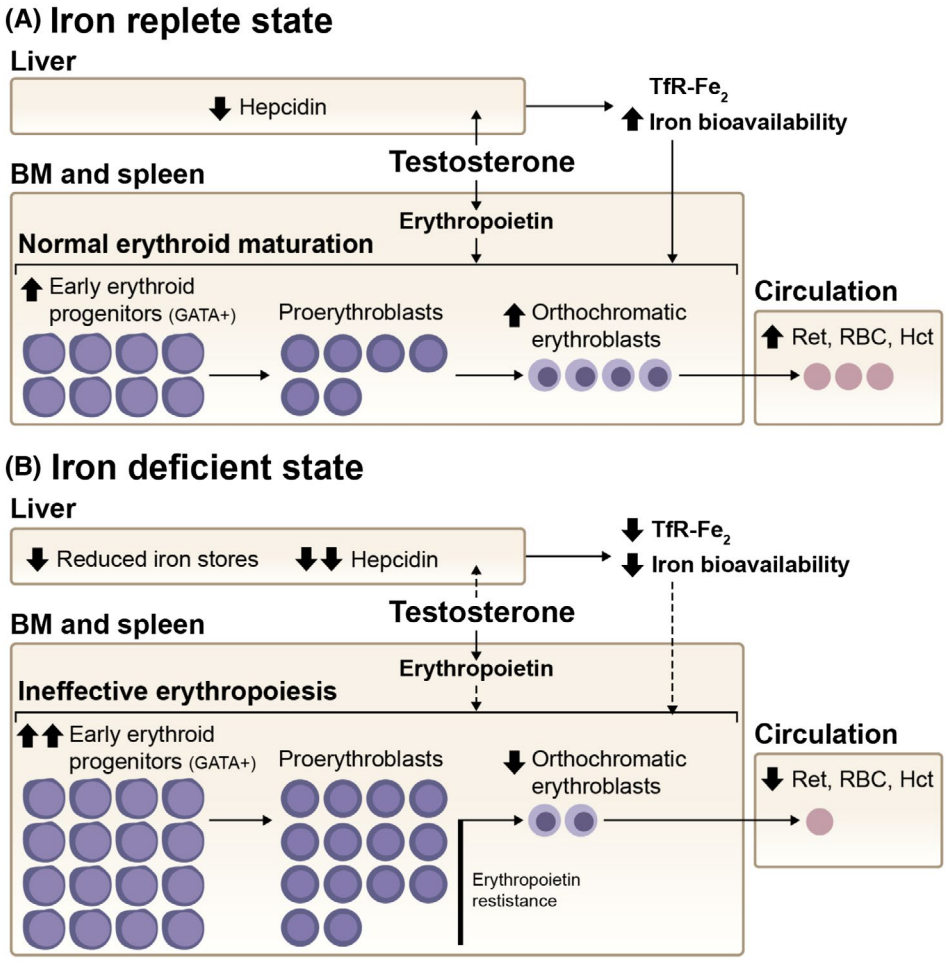

FIGURE 7 The current findings suggest that in the iron-replete state, testosterone increases the number of erythroid progenitors in the bone marrow and the spleen (the site of stress erythropoiesis in the anemic state) and stimulates iron-dependent erythropoiesis by increasing iron availability. Under the permissive action of erythropoietin, the expansion of early erythroblast pool induced by testosterone treatment is associated with increased number of orthochromatic (late) erythroblasts and circulating red blood cells. In iron-deficient state, testosterone treatment is associated with marked increase in the numbers of early GATA + erythroid progenitor cells as well as proerythroblasts. However, the ratio of the proerythroblasts to orthochromatic (late) erythroblasts is reduced by testosterone treatment due to ineffective erythropoiesis, likely because of erythropoietin resistance associated with iron deficiency. Thus, in a state of iron deficiency, the expansion of the erythropoietic progenitor pool during testosterone treatment is associated with ineffective erythropoiesis, resulting in a reduced number of circulating red blood cells

hematocrit by stimulating iron-dependent erythropoiesis.[22] Testosterone suppresses hepcidin transcription in men[3,12] as well as in mice,[21,22] upregulates ferroportin, and increases iron availability for erythropoiesis.[22] However, testosterone administration to hepcidin knock out mice is also associated with increases in hemoglobin and hematocrit.[23] Thus, although hepcidin suppression by testosterone increases iron availability for erythropoiesis, hepcidin suppression is not essential for mediating testosterone's effects on erythropoiesis in iron-replete healthy mice. The present study shows that testosterone stimulates erythropoiesis leading to an expansion of early erythropoietic progenitors. In a state of iron deficiency, the expansion of the erythropoietic progenitor pool by testosterone administration is associated with ineffective erythropoiesis, possibly due to a state of erythropoietin resistance induced by iron deficiency. Thus, iron availability, but not hepcidin, is essential for mediating testosterone's effects on erythropoiesis.

Although randomized trials of the effect of testosterone in in men with iron deficiency anemia have not been conducted two clinical case reports in humans support our findings.[44,45] In one open label trial of 24 patients with advanced breast cancer,[44] who were treated with androgens, four patients had low serum iron at baseline; androgen treatment further decreased serum iron and the expected increase in hemoglobin and hematocrit did not occur in response to androgen administration. Testosterone administration to one female patient with iron deficiency in this case report further decreased serum iron from 30 to 22 ug/dL and hemoglobin from 13.2 to 11.9 gm/dL[44]; the anemia was reversed after testosterone administration was discontinued and iron supplementation was provided.[44] In a second case,[45] administration of testosterone

to an iron-deficient male patient decreased serum iron from 10.8 to 6.0 ug/dL and hemoglobin from 7.5 to 6.0 g/dL (Gardner and Pringle). These case reports support our observations that in iron-deficient state, testosterone treatment worsens anemia.

These findings should be interpreted in the context of its limitations. Although the criteria for ineffective erythropoiesis were met (increased number of pro- and polychromatophilic erythroblasts, maturation block at the polychromatophilic erythroblasts stage, and increased LDH), only a very few cells in the spleen were cleaved caspase 3 positive, and therefore, we were not able to observe clear evidence of increased apoptosis in the spleen. We did observe an increase in the number of apoptotic cells in the bone marrow of iron-deficient mice.

These findings have clinical implications. Although testosterone treatment can correct unexplained anemia of aging and anemia of inflammation, it is important to evaluate the cause of anemia and rule out iron deficiency, before starting testosterone treatment because in these states, testosterone treatment could worsen anemia.

In summary, iron deficiency in mice is associated with ineffective erythropoiesis likely due to erythropoietin resistance. Testosterone treatment of iron-deficient mice worsens anemia by stimulating expansion of erythropoietic progenitors and ineffective erythropoiesis (Figure 7).

CONFLICT OF INTEREST

Dr. Bhasin reports receiving research grant funding, unrelated to the research reported in this manuscript, from NIA, NINR, NICHD, FNIH, AbbVie, Metro International Biotechnology, Alivegen, OPKO, Transition Therapeutics and FPT; receiving consultant fees from AbbVie, Aditumbio, and OPKO; and holding equity interest in FPT, LLC. He holds a patent on a method to calculate free testosterone concentration. These conflicts are overseen by and managed according to policies of the Office of Industry Interaction of the Mass General Brigham Health Care System. Other authors have reported no conflicts.

AUTHOR CONTRIBUTIONS

S. Bhasin, W. Guo, P. Schmidt, and M. Fleming designed the experiments; W. Guo performed the experiments; W. Guo, R. Abou Ghayda, and S. Bhasin wrote the paper. All authors reviewed the paper and provided critical feedback.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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