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RESEARCH ARTICLE

Transferrin receptor 2 (Tfr2) genetic deletion makes transfusion-independent a murine model of transfusion-dependent β-thalassemia

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Abstract

β-thalassemia is a genetic disorder caused by mutations in the β-globin gene, and characterized by anemia, ineffective erythropoiesis and iron overload. Patients affected by the most severe transfusion-dependent form of the disease (TDT) require lifelong blood transfusions and iron chelation therapy, a symptomatic treatment associated with several complications. Other therapeutic opportunities are available, but none is fully effective and/or applicable to all patients, calling for the identification of novel strategies. Transferrin receptor 2 (TFR2) balances red blood cells production according to iron availability, being an activator of the iron-regulatory hormone hepcidin in the liver and a modulator of erythropoietin signaling in erythroid cells. Selective *Tfr2* deletion in the BM improves anemia and iron-overload in non-TDT mice, both as a monotherapy and, even more strikingly, in combination with iron-restricting approaches. However, whether *Tfr2* targeting might represent a therapeutic option for TDT has never been investigated so far. Here, we prove that BM *Tfr2* deletion improves anemia, erythrocytes morphology and ineffective erythropoiesis in the $Hbb^{th1/th2}$ murine model of TDT. This effect is associated with a decrease in the expression of α-globin, which partially corrects the unbalance with β-globin chains and limits the precipitation of misfolded hemoglobin, and with a decrease in the activation of unfolded protein response. Remarkably, BM *Tfr2* deletion is also sufficient to avoid long-term blood transfusions required for survival of $Hbb^{th1/th2}$ animals, preventing mortality due to chronic anemia and reducing transfusion-associated complications, such as progressive iron-loading. Altogether, *TFR2* targeting might represent a promising therapeutic option also for TDT.

1 | INTRODUCTION

β-thalassemia is an autosomal recessive disorder due to mutations in the β-globin gene or in its promoter, that result in reduced or absent β-globin chain synthesis. The unbalance between α- and β-globin chains determines the accumulation of unstable and insoluble α-globin/heme complexes, called hemichromes, that precipitate, generate cytotoxic reactive oxygen species and lead to endoplasmic reticulum stress.[1–3] All these features affect the maturation and viability of erythroid precursors, resulting in ineffective erythropoiesis (IE), a tentative compensatory expansion of extramedullary erythropoiesis, that leads to anemia, splenomegaly, and decreased red blood cells (RBC) survival.[3]

The augmented proliferation of early erythroid precursors increases the production of the erythroid regulator erythroferrone (ERFE), a peptide hormone released in the circulation to inhibit the transcription of the iron-regulatory hormone hepcidin.[4,5] Suppression of hepcidin increases intestinal iron absorption and release from macrophages, resulting in progressive iron-loading, one of the most relevant complications in β-thalassemia.[6–8] Of note, increased iron uptake by erythroid cells further contributes to IE, enhancing heme and hemichrome production.[9–11]

Anemia severity can range from mild to severe and patients are classified as Non-Transfusion Dependent (NTDT) or Transfusion Dependent (TDT) based on their blood transfusion requirement.[12] Patients affected by severe TDT require lifelong blood transfusions and iron chelation to control iron-overload, symptomatic treatments that affect the quality of life.[3,13,14] For years, the only curative option was allogenic bone marrow (BM) transplantation, an approach effective in more than 90% of cases, but limited by the insufficient number of HLA-matched donors and, in some cases, associated to severe post-transplant complications.[15–17] Two promising innovative treatments, Activin Receptor Ligand Trap Luspatercept-Reblozyl[18,19] and the gene therapy product Zynteglo,[20–23] received FDA and EMA approval and EMA conditional marketing authorization for TDT patients, respectively: however, these are not fully effective in all patients and/or suitable for selected individuals.[24] For these reasons, the identification of novel therapeutic approaches, to be eventually combined with available therapies, is a clinical need.

Transferrin receptor 2 (TFR2), the hemochromatosis type III gene,[25] sensing the iron bound transferrin,[26] transcriptionally activates hepcidin in the liver.[27–29] The partially unknown mechanism involves the modulation of the Bone Morphogenetic Protein-Son of Mother Against Decapentaplegic (BMP-SMAD) signaling pathway.[30–34] TFR2 is a sensor of circulating iron also in erythroid cells, where it binds Erythropoietin (EPO) receptor (EPOR)[35] and inhibits the activation of the EPO-EPOR signaling.[36] Indeed, wild-type mice lacking BM *Tfr2* show enhanced erythropoiesis and increased RBC count, without changes in EPO levels.[36] Thus, TFR2 balances RBC production with systemic iron homeostasis, according to circulating iron levels. When transferrin saturation (TS) increases, it is stabilized on the plasma membrane of both hepatocytes, activating hepcidin to limit plasma iron flux,[37,38] and erythroblasts,[39] preventing excessive erythropoietic expansion. When TS decreases, TFR2 is shed from the plasma membrane[39] and/or degraded in lysosomes[40] enhancing both iron absorption and erythropoiesis to avoid anemia. However, the mechanism/s of the TFR2-mediated modulation of physiologic and ineffective erythropoiesis is still partially unknown and a current matter of investigation.

This function of TFR2 can be exploited for therapeutic purposes. Indeed, BM *Tfr2* deletion ameliorates anemia and ineffective erythropoiesis, and partially prevents hepatic iron loading in the NTDT $Hbb^{th3/+}$ murine model.[41] This effect can be enhanced through the simultaneous downregulation of the hepcidin inhibitor *Tmprss6*,[42,43] approach that improves the thalassemic phenotype inducing iron-restriction.[44,45]

Here, we aim at investigating whether TFR2 might represent a candidate therapeutic target not only for NTDT, but also for TDT, the form of the disease that requires regular blood transfusion treatment. We demonstrate that deletion of a single or both *Tfr2* allele in the BM of the $Hbb^{th1/th2}$ TDT murine model[46] ameliorates anemia and IE, partially preventing the age-dependent iron-loading, and completely abolishing blood transfusion requirement for survival.

2 | METHODS

2.1 | Animal models

$Hbb^{th1/+(47)}$ and $Hbb^{th2/+}$[48] mice (C57BL/6J background) were from the Jackson Laboratories, while $Tfr2^{+/-}$ mice (129S2 background) were as previously described.[49] Both $Hbb^{th1/+}$ and $Hbb^{th2/+}$ were crossed with $Tfr2^{+/-}$ mice obtaining $Tfr2^{+/-}/Hbb^{th1/+}$ and $Tfr2^{+/-}/Hbb^{th2/+}$ progenies on a mixed C57/129S2 background. $Tfr2^{+/-}/Hbb^{th1/+}$ and $Tfr2^{+/-}/Hbb^{th2/+}$ mice were then crossed and dated, to isolate day E14.5 embryos for fetal liver cells recovery and transplant. All animals were maintained in the animal facility at San Raffaele Scientific Institute, fed a regular rodent diet and under 12 h:12 h light: dark cycles. All animal studies were conducted under protocols approved by the Institutional Animal Care of San Raffaele Scientific Institute in accordance with the EU guidelines.

2.2 | Fetal liver transplantation and experimental design

Fetal liver cells (FLCs) isolated from day E14.5 C57/129S2 $Tfr2^{+/+}/Hbb^{th1/th2}$, $Tfr2^{+/-}/Hbb^{th1/th2}$ and $Tfr2^{-/-}/Hbb^{th1/th2}$ embryos were used for transplantation (FLT), as described.[46] In brief, 2×10^6 FLCs from $Tfr2^{+/+}/Hbb^{th1/th2}$, $Tfr2^{+/-}/Hbb^{th1/th2}$ and $Tfr2^{-/-}/Hbb^{th1/th2}$ embryos (expressing the CD45.2 surface antigen) were transplanted into lethally irradiated C57BL/6-Ly-5.1 male mice (expressing the CD45.1 surface antigen), generating $Hbb^{th1/th2}$ mice with both ($Tfr2^{BMWT}/Hbb^{th1/th2}$), a single ($Tfr2^{BMHet}/Hbb^{th1/th2}$) or no ($Tfr2^{BMKO}/Hbb^{th1/th2}$) *Tfr2* allele in the BM.

A cohort of mice was sacrificed eight weeks after FLT for phenotypic characterization, while a second cohort was maintained longer for the evaluation of blood transfusion requirement. Blood was collected by tail vein puncture for complete blood count (CBC) every four weeks, and weekly for monitoring hemoglobin (Hb) levels until 30 weeks after FLT, when mice were sacrificed. A blood transfusion was performed when Hb decreased to ≤5.5 g/dL. At sacrifice, blood was collected for CBC and serum iron, EPO and ERFE measurement; liver, spleen, kidneys and heart were weighed, dissected and snap-frozen immediately for RNA analysis, or dried for tissue iron quantification or processed for FACS analysis. BM cells were harvested and processed for flow cytometry or RNA analysis.

2.3 | Hematological analysis

CBC and reticulocytes percentage were determined on a IDEXX Procyte dx automated blood cell analyzer (Idexx Laboratories).

For Hb evaluation, a single drop of blood was collected and analyzed using the Hemoglobin colorimetric micro assay kit (Alpha Diagnostic International), following manufacturer's recommendations.

Blood smears were stained with Giemsa or Crystal Violet solution and pictures were captured using a Leica DM5000 microscope equipped with a Leica DFC480 digital camera.

Serum iron and total iron binding capacity (TIBC) were determined using The Total Iron Binding Capacity Kit (Randox Laboratories Ltd.), according to the manufacturer's instructions. Transferrin saturation was calculated as the ratio between serum iron and TIBC.

Serum EPO and ERFE levels were measured using mouse EPO quantikine set (R&D System) and Intrinsic Mouse Erythroferrone ELISA kit (Intrinsic Lifesciences) respectively, following standard protocol.

2.4 | Blood transfusions

Mice were transfused with 300 μl of freshly harvested blood from wild-type C57BL/6J male mice via retro-orbital injection, as previously described.[46] The last transfusion was performed two weeks before sacrifice.

2.5 | Tissue iron content

Tissue samples, dried at 65°C for 1 week, were weighed and digested in acid solution (3 M HCl, 0.6 M trichloroacetic acid) for 20 h at 65°C. Iron ammonium sulfate was dissolved in acid solution to generate a standard curve. The acid extracts were added to working chromogen reagent (1 volume of 0.1% bathophenanthroline sulfate and 1% thioglycolic acid solution, 5 volumes of water, and 5 volumes of saturated sodium acetate) and incubated for 30 min at room temperature. Then, the absorbance at 535 nm was determined for the quantification of iron content.

2.6 | Flow cytometry

BM and spleen cells were pre-treated with rat-anti-mouse CD16/CD32 (BD Pharmingen) in order to block unspecific Ig binding. Cells were subsequently incubated for 30 min in the dark at 4°C with FITC rat anti-mouse Ter119 and APC rat anti-mouse CD44 (BD Biosciences) for the evaluation of erythroid differentiation, or with FITC-conjugated anti-mouse CD45.1 and APC-conjugated anti-mouse CD45.2 antibodies (BD Biosciences) to monitor donor/host chimerism.

Analyses were performed using the FACS Canto™ II (BD Biosciences).

2.7 | Quantitative RT-PCR

RNA was extracted using the guanidinium thiocyanate–phenol–chloroform method (Trifast Reagent, Euroclone) following the standard protocol for snap-frozen liver, spleen and kidney samples. Fresh BM cells were lysed in Trifast reagent and then RNA was extracted combining the Trifast protocol followed by a resin-based purification (ReliaPrep RNA Miniprep system, Promega), after the addition of isopropanol.

cDNA was synthetized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to manufacturer's instructions. Real-time PCR analyses were performed on 7900HT Fast Real-Time PCR System (Applied Biosystems) using specific murine TaqMan Assays-on-Demand and Master Mix (Applied Biosystems) or specific murine oligos (designed using the NCBI Pick Primer software and generated by Merck) and SYBRgreen Master Mix (Applied Biosystems). RNA levels were normalized to the expression of the housekeeping gene Ribosomal Protein L13 *(Rpl13)* or β-Actin (*Actb*). Primers used are described in Table S1 and S2.

2.8 | Statistics

Data are presented as mean ± SE. Unpaired 2-tailed Student's t-test for multiple comparison or one-way ANOVA analysis was performed using GraphPad Prism 7.0 (GraphPad). $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | BM *Tfr2* deletion ameliorates anemia in the $Hbb^{th1/th2}$ model of transfusion-dependent β-thalassemia

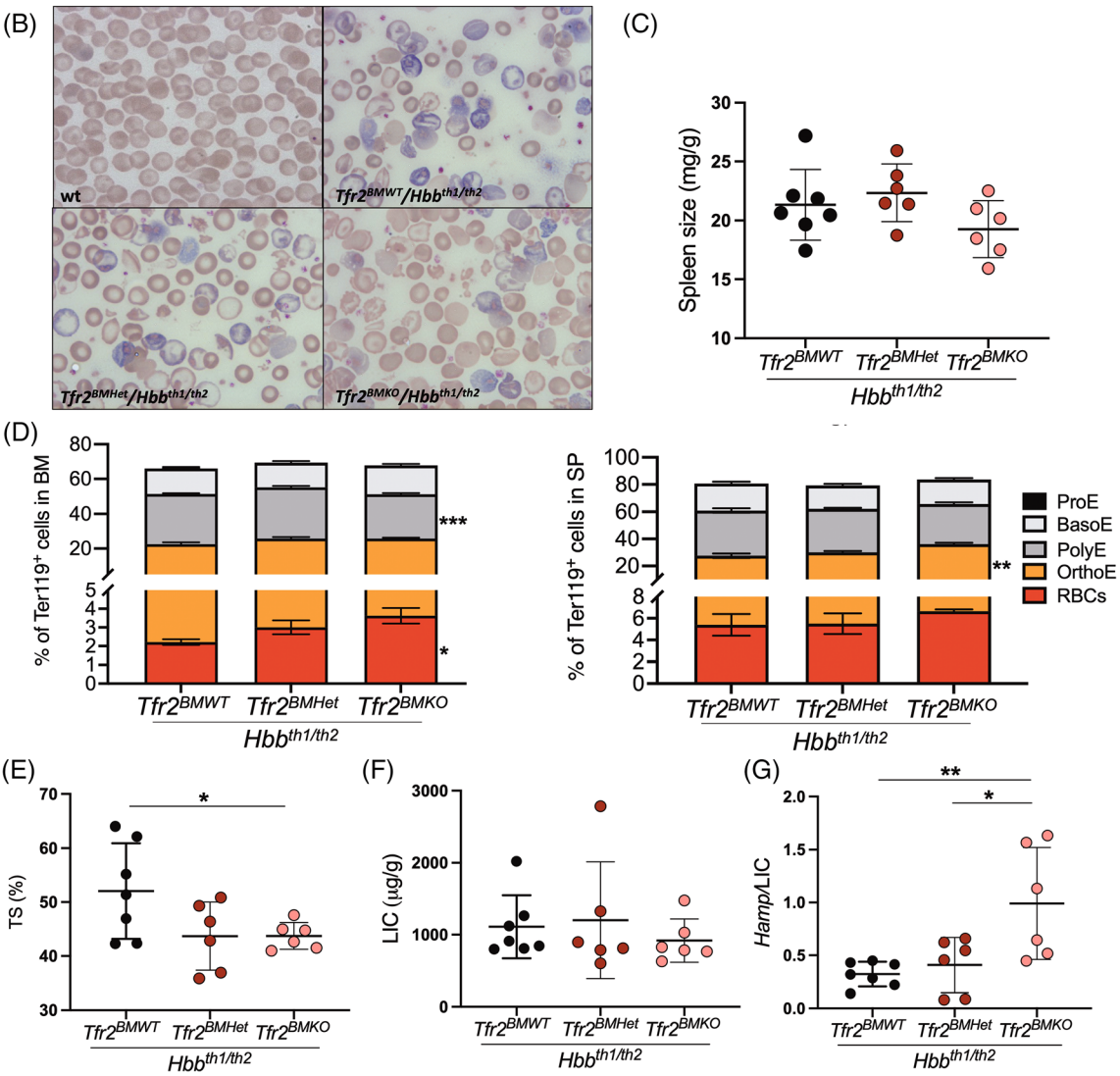
As we shown previously, myeloablated wild-type (WT) animals transplanted with FLCs from $Hbb^{th1/th2}$ embryos develop a severe anemia (Figure 1A), requiring blood transfusion for survival.[46] To evaluate the effect of *Tfr2* deletion on the phenotype of these animals, we engrafted FLCs from $Hbb^{th1/th2}$ embryos carrying deletion of both

($Tfr2^{BMKO}/Hbb^{th1/th2}$) or a single *Tfr2* allele ($Tfr2^{BMHet}/Hbb^{th1/th2}$) into myeloablated WT animals. As controls, we used mice transplanted with FLCs from $Hbb^{th1/th2}$ embryos harboring both *Tfr2* alleles ($Tfr2^{BMWT}/Hbb^{th1/th2}$).

A cohort of mice was sacrificed and analyzed eight weeks after FLT, before the onset of blood transfusion requirement, for a complete phenotypic characterization. At this time-point, mice of the three genotypes were phenotypically undistinguishable with comparable body weight (Figure S1A). Chimerism was >95% and >85% in the BM and in the spleen (Figure S1B,C), respectively, in all animals. The expression of *Tfr2* was reduced in agreement with the genotype of the transplanted FLCs in both organs (Figure S1D,E).

(A)

	$Tfr2^{BMWT}/Hbb^{th1/th2}$	$Tfr2^{BMHet}/Hbb^{th1/th2}$	$Tfr2^{BMKO}/Hbb^{th1/th2}$
RBC (*10^6/μl)	6.53±0.10	7.44±0.13****	8.57±0.17****,####
Hb (g/dL)	7.69±0.11	8.65±0.18****	9.61±0.19****,##
Hct (%)	29.56±0.37	32.39±0.54***	36.07±0.67****,###
MCV (fL)	45.28±0.27	43.55±0.30***	42.14±0.31***,##
MCH (pg)	11.77±0.06	11.62±0.13	11.21±0.04***,##
Retics (%)	70.92±0.90	64.28±2.85*	60.00±1.83***
PLT (*10^3/μl)	1211.0±52.9	1264.8±43.8	1180.3±61.4
WBC (*10^3/μl)	56.72±4.03	67.36±6.182	41.42±4.44*,##



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Tfr2^{BMKO}/*Hbb*$^{th1/th2}$ mice had increased RBC count, Hb levels and hematocrit (Hct) relative to *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ controls, and decreased percentage of reticulocytes (Retics), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and white blood cells (WBC) (Figure 1A). This reduction in WBC may indicate less nucleated red cells in circulation, since nucleated erythroid cells are recognized as WBC. *Tfr2* haploinsufficient animals showed an intermediate phenotype, as in NTDT mice.[41] Increased Hb was accompanied by improved RBC morphology (Figure 1B), while splenomegaly was not corrected (Figure 1C), as in our previous studies.[41,42]

At eight weeks after FLT, complete BM *Tfr2* deletion attenuated IE. In the BM, comparing *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ to *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ mice, the percentage of polychromatic erythroblasts was decreased and that of mature RBCs was increased, whereas in the spleen, an increased proportion of orthochromatic erythroblasts was observed (Figure 1D).

In agreement, and in line with increased Hb, kidney *Epo* mRNA levels (Figure 2A) and serum EPO levels (Figure 2B) were lower in *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice as compared to controls, with *Tfr2* haploinsufficient *Hbb*$^{th1/th2}$ animals showing intermediate serum EPO levels. EPO reduction, together with decreased immature erythroid precursors, limited the production of the erythroid regulator Erythroferrone (*Erfe*) in the BM (Figure 2C), but not in the spleen (Figure 2D) with a net decrease in circulating levels (Figure 2E) in *Tfr2* deficient animals, in agreement with the increased hepatic *Hamp* expression.

The expression levels of other EPO-EPOR target genes as B-cell lymphoma-extra large[51] (*Bcl-xl*, Figure S2A,B) and *Epor* itself[52,53] (Figure S2C,D) were similar in animals of the 3 genotypes both in the BM and in the spleen. However, *Erfe*, *Bcl-xl* and *Epor* levels relative to serum EPO were higher in *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ than in controls both in the BM (Figure 2F–H) and in the spleen (Figure 2I–K), confirming that *Tfr2* deletion boosts EPO sensitivity also in this severe model, as shown previously.[36,41]

3.2 | The erythroferrone-mediated hepcidin inhibition is blunted in *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice

Tfr2^{BMKO}/*Hbb*$^{th1/th2}$ mice showed a reduction of transferrin saturation (Figure 1E) and a trend toward lower serum iron (Figure S1F) as compared to *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ controls, likely as a result of increased iron consumption by the enhanced erythropoiesis. The iron content in the liver (LIC, Figure 1F), spleen (SIC, Figure S1G), kidney (KIC, Figure S1H) and heart (HIC, Figure S1I) was comparable among animals of the three genotypes. In line with these results, hepatic expression of Transferrin Receptor 1 (*Tfr1*, Figure S1J) and of the iron-responsive Bone Morphogenetic Protein 6[50] (*Bmp6*, Figure S1K) were unchanged among the three genotypes. However, Hepcidin (*Hamp*) mRNA levels were higher in the liver of *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice relative to *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$, both as absolute values (Figure S1L), and when values are normalized on LIC (Figure 1F), in line with a reduced hepcidin inhibition mediated by the more effective erythropoiesis. High hepcidin levels might contribute to lowering transferrin saturation in *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice.

3.3 | BM *Tfr2* deletion reduces α-globin transcription and unfolded protein response in erythroid tissues of *Hbb*$^{th1/th2}$ mice

Irrespective of the *Tfr2* genotype, the *Hbb*$^{th1/th2}$ model had very low expression of the β-major globin allele relative to wt (Figure S2E), accompanied by a tentative compensatory increase in the expression of the β-minor allele (Figure S2F), in line with previous results.[47] The mRNA levels of α-globin were high in *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ animals relative to wt (Figure 3A), to likely counteract the low hemoglobin, but finally worsening the unbalance between α and β-globin chains, the key driver of IE in β-thalassemia.

The accumulation of free α-globin chains can be counteracted by decreasing α-globin[54] or increasing the expression of γ-globin and the production of fetal hemoglobin.[55] We demonstrated that BM *Tfr2* deletion was associated with reduced mRNA levels of α-globin, both as absolute value (Figure 3A), and as a ratio over the expression of the α-hemoglobin stabilizing protein (*Ahsp*, Figure S2G), a chaperone required to prevent the harmful aggregation of α-globin.[56,57] This

FIGURE 1 **Bone marrow *Tfr2* deletion ameliorates anemia, red blood cells morphology and ineffective erythropoiesis of transfusion-dependent *Hbb*$^{th1/th2}$ mice**. *Hbb*$^{th1/th2}$ mice with inactivation of both (*Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$) or a single (*Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$) *Tfr2* allele in the bone marrow were generated through fetal liver cells transplantation (FLT). Complete blood count [red blood cell (RBC) count, hemoglobin (Hb) levels, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), reticulocytes (Retics), platelet (PLT) and white blood cell (WBC) count] was evaluated 8 weeks after FLT showing anemia improvement upon *Tfr2* loss [(A), mean values of 12/14 animals/genotype ± standard errors]. A cohort of *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$, *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ and *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice was sacrificed at this time point for a full phenotypic characterization. *Tfr2* deletion in the bone marrow (BM); (B) ameliorated RBCs morphology (representative pictures of giemsa staining of blood smears, original magnification 100X); (C) did not correct splenomegaly (spleen size relative to body weight); (D) improved erythroid differentiation [percentage of Ter119^{+} cells on total alive cells and subpopulation composition. Gated Clusters: proerythroblasts (I), basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts and immature reticulocytes (IV), and mature red cells (V)] both in the BM and in the spleen (SP); (E) reduced Transferrin saturation (TS); (F) did not modify liver iron content (LIC); (G) increased hepcidin (*Hamp*) levels relative to hepatic iron-loading [ratio between hepatic *Hamp* mRNA levels relative to Ribosomal protein l13 (*Rpl13*) and LIC]. Bars represent standard error (SE). Asterisks refer to statistically significant differences versus *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ mice (A), (D) or as indicated. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Hashtags refer to statistically significant differences versus *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ mice (A). $^{\#\#}p < 0.005$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$.

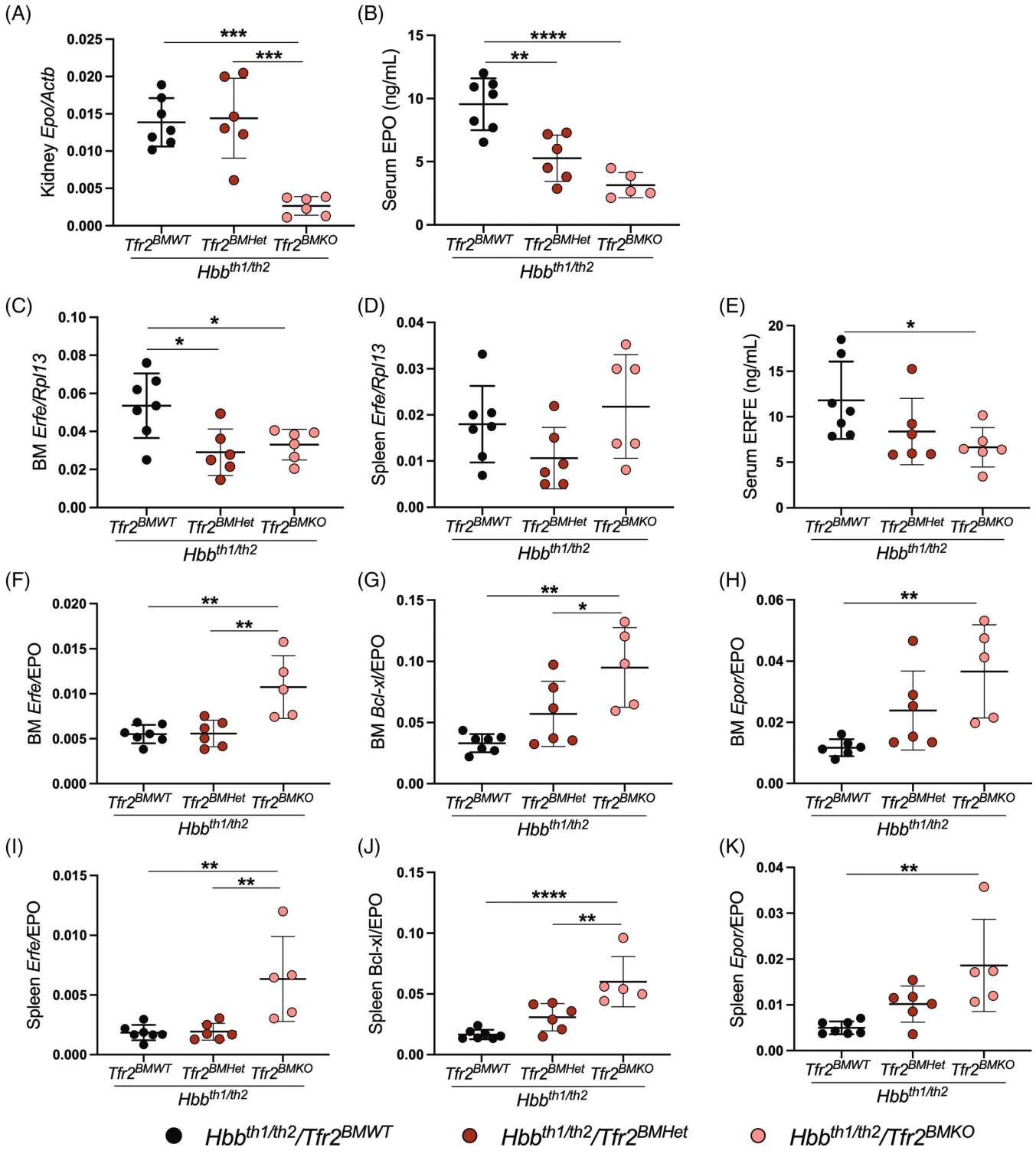
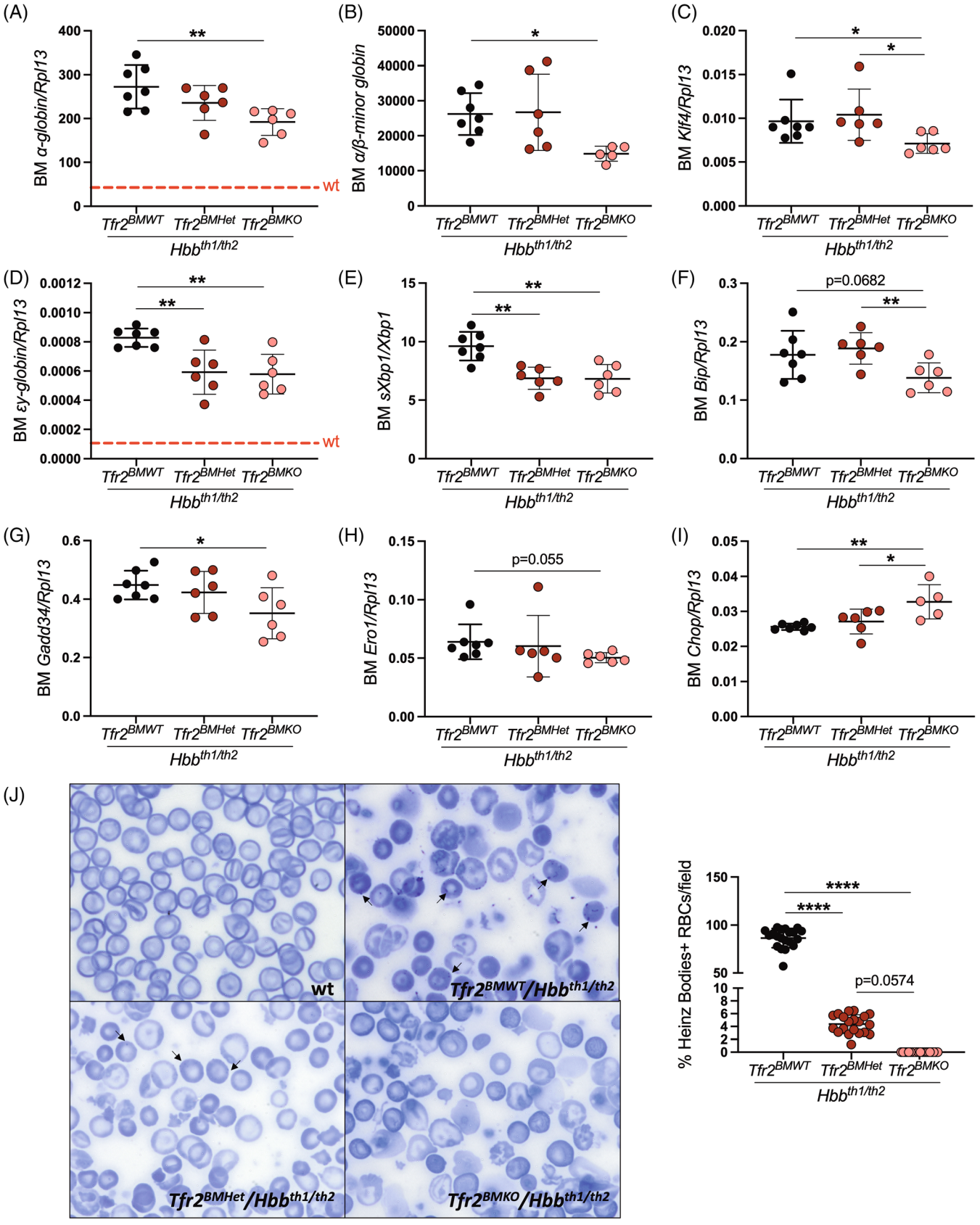


FIGURE 2 **Bone marrow *Tfr2* deletion enhances erythropoietin sensitivity in erythroid tissues of *Hbb*$^{th1/th2}$ mice**. Eight weeks after fetal liver transplantation erythropoietin (*Epo*) levels were reduced in *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice both in the kidney (A) and in the serum (B), decreasing erythroferrone (*Erfe*) in the bone marrow (BM) and in the serum, but not in the spleen (C)–(E). The transcription of EPO target genes normalized on circulating EPO levels was enhanced both in the BM and in the spleen of *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice. In the figure are reported: kidney mRNA levels of *Epo* relative to β-Actin (*Actb*) (A); serum EPO levels (B); *Erfe* mRNA levels of relative to Ribosomal Protein L13 (*Rpl13*) in the bone marrow (BM, (C)) and in the spleen (D); serum ERFE levels (E); the ratio between BM mRNA levels of *Erfe*, B-cell lymphoma-extra large (*Bcl-xl*) and Erythropoietin receptor (*Epor*) relative to Ribosomal Protein L13 (*Rpl13*) and EPO both in the BM (F),(G),(H) and in the spleen (I),(J),(K). Bars represent standard error (SE). Asterisks refer to statistically significant differences. *$p < 0.05$; **$p < 0.005$; ****$p < 0.0001$. [Color figure can be viewed at wileyonlinelibrary.com]



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suggests that, in the absence of *Tfr2*, α-globin was less produced and less toxic, because of the more appropriate levels of *Ahsp*, pointing to a restored unbalance between α and β-globin chains. In agreement, the ratio between α and β-minor globin chains was lower in $Tfr2^{BMKO}/Hbb^{th1/th2}$ animals as compared to $Tfr2^{BMWT}/Hbb^{th1/th2}$ controls (Figure 3B).

This could be explained by an *in vivo* selection of erythroid cells with less unbalanced synthesis of the globin chains or, more intriguingly, by a direct inhibitory effect on α-globin transcription mediated by *Tfr2* deficiency. Interestingly, the mRNA levels of the transcription factor Kruppel Like Factor 4 (*Klf4*), key player in the induction of α-globin expression,[58,59] were reduced in the BM of $Tfr2^{BMKO}/Hbb^{th1/th2}$ mice relative to controls (Figure 3C). This raised the possibility that TFR2 might directly modulate α-globin via KLF4, but the underlying mechanism remained to be clarified. We found that also the expression of the late murine embryonic globin chain εγ is reduced in the BM of $Tfr2^{BMKO}/Hbb^{th1/th2}$ mice (Figure 3D), suggesting that KLF4 may be involved also in the transcriptional control of εγ-globin, in analogy with the KLF4-mediated common modulation of α and γ-globin genes in humans.[60]

To analyze more in depth the changes induced by *Tfr2* deficiency in thalassemic BM cells, we focused on the endoplasmic reticulum stress, a critical consequence of free α-globin and hemichrome precipitation, that we found alleviated in $Tfr2^{BMKO}/Hbb^{th1/th2}$ mice. Indeed, the expression of several genes activated by the UPR pathways was decreased. In detail, we observed a reduction in the proportion between the spliced and unspliced forms of X-box DNA Binding Protein, UPR-specific b-ZIP transcription factor[61] (*sXbp1/Xbp1*, Figure 3E), due to a diminished expression of *sXbp1* (Figure S2H) in the absence of changes in total *Xbp1* levels (Figure S2I). Moreover, mRNA levels of Binding Immunoglobulin Protein[62] (*Bip*, Figure 3F), Growth Arrest and DNA Damage-inducible protein 34[63] (*Gadd34*, Figure 3G) and Endoplasmic Reticulum Oxidoreductase 1[64] (*Ero1*, Figure 3H) were reduced in the BM of $Tfr2^{BMKO}/Hbb^{th1/th2}$ relative to $Tfr2^{BMWT}/Hbb^{th1/th2}$ and/or $Tfr2^{BMHet}/Hbb^{th1/th2}$ mice. Surprisingly, the expression of C/EBP Homologous Protein (*Chop*)[65,66] was increased in the BM of *Tfr2* deficient animals (Figure 3I). Since CHOP is an EPO-EPOR target gene, its upregulation in BM-derived cells is in agreement with the inappropriately high EPO-EPOR signaling due to *Tfr2* genetic inactivation.

Overall, these findings suggest that, in the absence of *Tfr2*, thalassemic erythroid cells have a reduced abundance of toxic free α-globin chains. This, together with the iron-restricted phenotype of *Tfr2*-deficient erythroid cells, evident by low MCV and MCH (Figure 1A), results in reduced hemichromes precipitation, as shown by the drastic decrease in the presence of Heinz bodies within $Tfr2^{BMKO}/Hbb^{th1/th2}$ RBCs (Figure 3J), and in line with the improved erythrocytes morphology (Figure 1B).

3.4 | Blood transfusion requirement is completely abolished in $Hbb^{th1/th2}$ mice lacking BM *Tfr2*

The beneficial effect of *Tfr2* deletion was maintained over time. $Tfr2^{BMWT}/Hbb^{th1/th2}$ mice became progressively more transfusion-dependent overtime, showing Hb levels of 5.5 g/dL at 14 weeks post FLT. RBC count (Figure 4A), Hb levels (Figure 4B) and Hct (Figure 4C) were maintained stable by blood transfusions in $Tfr2^{BMWT}/Hbb^{th1/th2}$ animals for the entire follow-up, while reticulocytes were progressively reduced (Figure 4D), likely due to transfusion-mediated inhibition of erythropoiesis.[67,68] In line with the reduced percentage of reticulocytes, MCV decreased during the 30-week-long period (Figure 4E). Since transfusions were interrupted at 28 weeks to minimize the confounding effect of administered blood in the erythropoietic phenotype of $Tfr2^{BMWT}/Hbb^{th1/th2}$ mice, the increase in reticulocytes and MCV observed at the time of the sacrifice likely reflected recovery of endogenous erythropoiesis. MCH increased during follow-up (Figure 4F) because of the progressive substitution of thalassemic RBCs with wild-type ones.

$Tfr2^{BMHet}/Hbb^{th1/th2}$ mice maintained higher RBC count and Hct for the entire follow-up and Hb until 16 weeks, when Hb reached values of transfused $Tfr2^{BMWT}/Hbb^{th1/th2}$ animals. $Tfr2^{BMKO}/Hbb^{th1/th2}$ mice showed RBC count, Hb levels and Hct higher than $Tfr2^{BMWT}/Hbb^{th1/th2}$ and $Tfr2^{BMHet}/Hbb^{th1/th2}$ mice for the entire 30-week-long period (Figure 4A–C). Reticulocytes count was reduced in mice lacking both BM *Tfr2* alleles, but not in haploinsufficient animals (Figure 4D). MCV and MCH were decreased in a comparable manner in both $Tfr2^{BMKO}/Hbb^{th1/th2}$ and $Tfr2^{BMHet}/Hbb^{th1/th2}$ mice, indicating the development of an iron-restricted erythropoiesis, as previously shown in $Tfr2^{BMKO}/Hbb^{th3/+}$ animals.[41,42] Of note, $Tfr2^{BMWT}/Hbb^{th1/th2}$ mice required transfusion of an average 107.34 ± 15.36 μl of

FIGURE 3 **Bone marrow *Tfr2* deletion reduces the expression of α-globin and of target genes of the unfolded protein response in the bone marrow of $Hbb^{th1/th2}$ mice**. Eight weeks after fetal liver transplantation $Tfr2^{BMKO}/Hbb^{th1/th2}$ mice showed decreased expression of α-globin in the bone marrow (BM, (A)), which partially corrected the unbalance with β-globin (B). The expression levels of the transcription factor Kruppel like factor 4 (*Klf4*, (C)), of the fetal globin εγ (D) and of several genes activated by the unfolded protein response (E)–(H) were reduced, with the exception of C/EBP Homologous Protein (*Chop*, (I)) which was increased. Heinz bodies in red blood cells (RBC) were decreased in mice lacking BM *Tfr2* (J). In the figure are represented: BM mRNA levels of α-globin (A), α- over β-minor globin (B), *Klf4* (C), εγ-globin (D), spliced over unspliced X-box DNA Binding Protein, UPR-specific b-ZIP transcription factor (*sXbp1/Xbp1*, (E)), Binding immunoglobulin protein (*Bip*, (F)), Growth arrest and DNA damage-inducible protein 34 (*Gadd34*, (G)), Endoplasmic Reticulum Oxidoreductase 1 (*Ero1*, (H)) and *Chop* (I) relative to Ribosomal Protein L13 (*Rpl13*); representative pictures of Crystal Violet staining of blood smears (Original magnification 100X) and the quantification of the percentage of RBCs carrying Heinz bodies performed in 10 different fields/animal (J). Dotted red lines indicate mean values wild-type (wt) mice. Bars represent standard error (SE). Asterisks refer to statistically significant differences. *$p < 0.05$; **$p < 0.005$; ****$p < 0.0001$. P-values close to 0.05 were also indicated.

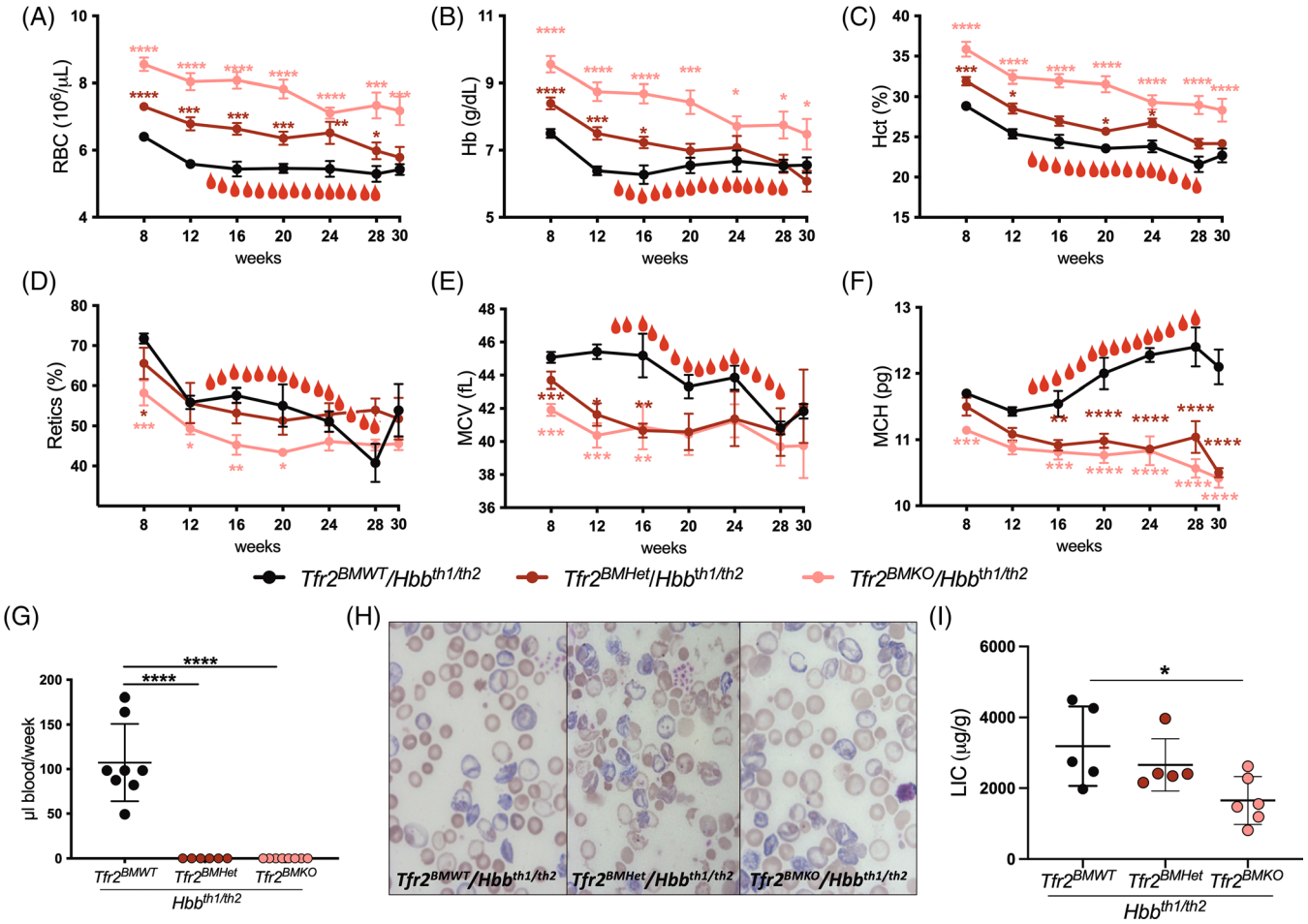

FIGURE 4 **Bone marrow *Tfr2* deletion abolishes blood transfusion dependence of *Hbb*$^{th1/th2}$ mice.** Complete blood count was determined every 4 weeks in a cohort of *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$, *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ and *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice until sacrifice 30 weeks after fetal liver transplantation (FLT). Hemoglobin (Hb) levels were monitored weekly and blood transfusion was performed when Hb ≤ 5.5 g/dL. Anemia was improved in mice lacking bone marrow (BM) *Tfr2* for the entire time-span (A)–(F), abolishing blood transfusion requirement for survival (G). Erythrocytes morphology was improved (H) in *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice and liver iron content (LIC) decreased (I). In the figure are represented: red blood cell count (RBC, (A)), Hb levels (B), hematocrit (Hct, (C)), reticulocytes percentage (Retics, (D)), mean corpuscular volume (MCV, (E)), mean corpuscular hemoglobin (MCH, (F)), average blood transfused per week (G), representative pictures of Giemsa-stained blood smears (original magnification 100X, (H)) and LIC of animals at 30 weeks after FLT (I). Red drops indicate time points in which at least 1 *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ animal received blood transfusion. Bars represent standard error (SE). Asterisks refer to statistically significant differences. **p* < 0.05; ***p* < 0.005; ****p* < 0.0005; *****p* < 0.0001.

blood/animal/week throughout the entire follow-up, while animals with both heterozygous and homozygous BM *Tfr2* deletion did never require transfusions for survival (Figure 4G).

The beneficial effect of *Tfr2* inactivation on thalassemic RBCs morphology was maintained also at this late time-point, as evident from the comparison between *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ and *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ blood smears, while *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ animals showed mostly RBCs derived from blood transfusions (Figure 4H).

3.5 | BM *Tfr2* deletion reduces hepatic iron-loading of aged *Hbb*$^{th1/th2}$ mice

Despite the consistent anemia improvement, erythroid differentiation did not show striking differences among the three genotypes both in the BM and in the spleen (Figure S3A). This is likely due to the suppression of ineffective and expanded erythropoiesis induced by blood transfusions[67,68] in *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ mice. However, a reduction in the proportion of polychromatophilic erythroblasts and an increase in mature RBCs can still be detected in the spleen of *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice as compared to *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ (Figure S3A), which, in line with previous findings,[41,42] is not paralleled by splenomegaly reduction (Figure S3B).

Also, at this late time-point *Epo* production from the kidney was reduced in both *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ and *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ mice as compared to *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ (Figure S3C), compatible with improved anemia.

At difference with data from mice eight weeks after FLT, at this time point, *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice showed lower LIC (Figure 4I) and a trend toward reduced SIC (Figure S3D) when compared with both

Tfr2^{BMWT}/*Hbb*$^{th1/th2}$ and *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ animals, proving that BM *Tfr2* inactivation may prevent the age-dependent iron-loading in a model of TD-thalassemia. Irrespective of blood transfusions, LIC was not higher in *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ as compared to *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ mice (Figure 4I), likely because of the mild transfusional regimen applied.

4 | DISCUSSION

The therapy of TDT patients is still suboptimal. Recent advances in the elucidation of the pathophysiological mechanisms of the disease led to the design of novel therapeutic approaches.[14,69] However, none of these is fully effective in all patients, even when the genetic defect is corrected (i.e., by gene therapy), likely because different modifiers contribute to the complex phenotype of patients. For this reason, the identification of novel strategies to be eventually combined with available treatments, as occurs in hematological cancers, might offer new therapeutic perspectives.

Our recent studies identified TFR2 as a potential promising target for the treatment of NTD β-thalassemia.[41,42] However, its effectiveness in the most severe, transfusion dependent form of the disease was not yet addressed. Here, we prove that, despite the severity of the genetic defect, hematopoietic *Tfr2* deletion ameliorates ineffective erythropoiesis, anemia and iron-overload also in a TDT murine model. This improvement is associated to a substantial increase in transfusion-free survival of both *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ and *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice, which are transfusion-independent 16 weeks after the onset of blood transfusion requirement in *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ animals. Besides Luspatercept, this is the sole approach not correcting the genetic defect, which revealed able to reduce blood transfusion requirement in a TDT murine model. In a clinical perspective, we can speculate that our strategy might lead to a complete transfusion independence in milder patients, and to a significant decrease in blood requirement in more severe patients.

In line with our previous findings,[36,41,42] BM *Tfr2* deletion does not directly influence systemic iron homeostasis, indeed both tissue and circulating iron levels are comparable among animals of the three genotypes eight weeks after FLT. However, hepcidin expression is higher in *Tfr2*-deficient animals despite comparable LIC, likely because of the partial correction of IE and the consequent reduction of ERFE production. On a long-term, this reduces dietary iron absorption and, in association with the higher iron consumption by the more effective erythropoiesis, prevents hepatic iron-loading with aging.

Surprisingly, hepatic iron levels were comparable between untransfused *Tfr2*^{BMHet}/*Hbb*$^{th1/th2}$ and regularly transfused *Tfr2*^{BMWT}/*Hbb*$^{th1/th2}$ animals: this is likely due to the mild transfusion protocol utilized that provided mice with the minimal amount of blood required for maintaining Hb levels at about 6 g/dL. If a heavier or more prolonged transfusion regimen is applied, further worsening iron loading, the beneficial effect of BM *Tfr2* deletion, which abolishes blood transfusion requirement, would be even more impressive.

We previously demonstrated that *Tfr2* deletion enhances EPO sensitivity of erythroid cells in both wild-type mice[36] and in a NTDT model,[41] and here confirm that this occurs in a TDT model, too. However, this finding does not completely explain the mechanism responsible for the TFR2-mediated improvement of thalassemic erythropoiesis. In the attempt of unravel this point, we found that the BM expression of α-globin is reduced, likely through the inhibition of the transcription factor *Klf4*.[58,59] Of note, we observed a minimal decreased expression also of εγ-globin chain, excluding that *Tfr2* inactivation improves erythropoiesis reactivating fetal hemoglobin. This inhibition of εγ-globin transcription is unlikely to negatively impact on hemoglobin synthesis, since the strong effect on α-globin, and might occur through the downmodulation of *Klf4* as for human γ-globin.[60]

Low α-globin transcription and iron-restricted erythropoiesis, as indicated by low MCV and MCH in *Tfr2*^{BMKO}/*Hbb*$^{th1/th2}$ mice, contribute to decrease hemichrome formation and precipitation,[11,54] as supported by the improved RBC morphology and by reduced presence of denatured hemoglobin in *Tfr2*-deficient animals. This limits toxicity associated to excess α-globin and minimizes the endoplasmic reticulum stress due to unfolded protein response, as shown by a diminished expression of several UPR target genes. Surprisingly, *Chop* mRNA levels are even increased in the BM of *Tfr2* deficient animals, claiming for a UPR-independent regulation. Since previous data suggested that EPO can directly activate *Chop* transcription, thus promoting hemoglobinization[70,71] and that *Chop* deletion in vascular smooth muscle cells activates *Klf4* transcription,[72] we propose that in our model *Tfr2* deletion, increasing EPO signaling, activates *Chop* transcription, that, in turn, limits the expression of *Klf4* and α-globin, partially correcting the α:β-globin chains unbalance and improving erythroid differentiation and hemoglobinization.

The missing point is the lack of a tool to pharmacologically inhibit TFR2. We tested specific antisense oligonucleotides (IONIS Pharmaceuticals), which, despite effective in erythroid cells *in* vitro, failed in downregulating *Tfr2* expression in erythroid tissues when systemically administered in vivo (unpublished data), possibly because of poor cellular uptake.[73,74] In the future, the introduction of specific modifications for erythroid targeting or encapsulation into nanoparticles/liposomes for BM delivery[75,76] could represent an approach to solve this challenging issue.

Overall, our findings demonstrate that TFR2 targeting represents a new promising therapeutic opportunity for the management of β-thalassemia, able to strongly reduce blood transfusion requirement, worth to be tested both as a monotherapy and in association with available treatments.

AUTHOR CONTRIBUTIONS

Simona Maria Di Modica performed research, analyzed results and contributed to paper writing; Emanuele Tanzi, Violante Olivari, Maria Rosa Lidonnici, Mariateresa Pettinato, Alessia Pagani, Francesca Tiboni and Valeria Furiosi performed experiments; Laura Silvestri contributed to critical data analysis; Giuliana Ferrari participated to data analysis and paper editing; Stefano Rivella provided the thalassemia major murine model and edited the paper; Antonella Nai conceived the experiments, analyzed results, wrote and reviewed the manuscript. All Authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

Antonella Nai received research funding from Celgene (BMS group). Stefano Rivella is a member of scientific advisory board of Ionis Pharmaceuticals, Meira GTx, Incyte, Vifor and Disc Medicine and owns stock options from Disc Medicine. Stefano Rivella has been or is consultant for Cambridge Healthcare Res, Celgene Corporation, Catenion, First Manhattan Co., FORMA Therapeutics, Ghost Tree Capital, Keros Therapeutics, Noble insight, Protagonist Therapeutics, Sanofi Aventis U.S., Slingshot Insight, Techspert.io and BVF Partners L.P., Rallybio, LLC, venBio Select LLC. The other authors declare no financial conflict-of-interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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