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RESEARCH ARTICLE

Bone marrow *Tfr2* deletion improves the therapeutic efficacy of the activin-receptor ligand trap RAP-536 in β-thalassemic mice

Emanuele Tanzi[1,2] | Simona Maria Di Modica[1] | Jessica Bordini[2,3] | Violante Olivari[1,2] | Alessia Pagani[1] | Valeria Furiosi[1] | Laura Silvestri[1,2] | Alessandro Campanella[2,3] | Antonella Nai[1,2]

[1]Regulation of Iron Metabolism Unit, Division of Genetics and Cell Biology, IRCCS Ospedale San Raffaele, Milan, Italy

[2]Vita-Salute San Raffaele University, Milan, Italy

[3]B-cell Neoplasia Unit, Division of Experimental Oncology, IRCCS Ospedale San Raffaele, Milan, Italy

Correspondence
Antonella Nai, IRCCS Ospedale San Raffaele, Via Olgettina, 58, 20132 Milan, Italy.
Email: nai.antonella@hsr.it

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Abstract

β-thalassemia is a disorder characterized by anemia, ineffective erythropoiesis (IE), and iron overload, whose treatment still requires improvement. The activin receptor-ligand trap Luspatercept, a novel therapeutic option for β-thalassemia, stimulates erythroid differentiation inhibiting the transforming growth factor β pathway. However, its exact mechanism of action and the possible connection with erythropoietin (*Epo*), the erythropoiesis governing cytokine, remain to be clarified. Moreover, Luspatercept does not correct all the features of the disease, calling for the identification of strategies that enhance its efficacy. Transferrin receptor 2 (TFR2) regulates systemic iron homeostasis in the liver and modulates the response to *Epo* of erythroid cells, thus balancing red blood cells production with iron availability. Stimulating *Epo* signaling, hematopoietic *Tfr2* deletion ameliorates anemia and IE in $Hbb^{th3/+}$ thalassemic mice. To investigate whether hematopoietic *Tfr2* inactivation improves the efficacy of Luspatercept, we treated $Hbb^{th3/+}$ mice with or without hematopoietic *Tfr2* ($Tfr2^{BMKO}/Hbb^{th3/+}$) with RAP-536, the murine analog of Luspatercept. As expected, both hematopoietic *Tfr2* deletion and RAP-536 significantly ameliorate IE and anemia, and the combined approach has an additive effect. Since RAP-536 has comparable efficacy in both $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ animals, we propose that the drug promotes erythroid differentiation independently of TFR2 and EPO stimulation. Notably, the lack of *Tfr2*, but not RAP-536, can also attenuate iron-overload and related complications. Overall, our results shed further light on the mechanism of action of Luspatercept and suggest that strategies aimed at inhibiting hematopoietic TFR2 might improve the therapeutic efficacy of activin receptor-ligand traps.

1 | INTRODUCTION

β-thalassemia is an inherited recessive disorder due to mutations in the β-globin gene or in its promoter, characterized by ineffective erythropoiesis (IE), chronic anemia, and secondary iron overload. Different mutations lead to a variable degree of reduction of β-globin chain synthesis, resulting in a heterogeneous severity of the disease.[1–6] Mild anemia and lack of need for regular blood transfusion regimen are features of non-transfusion-dependent β-thalassemia (NTDT). Patients affected by the most severe transfusion-dependent (TDT) form require lifelong regular blood transfusions for survival, a suboptimal and demanding therapeutic approach[2,7,8] that should be associated to iron chelation to avoid excessive iron accumulation.[9–14] The only curative options for TDT are hematopoietic stem-cell transplantation and gene therapy, both expensive approaches limited by the paucity of HLA-matched donors and the complexity of technical issues for cell products, respectively.[15–20] Promising innovative strategies are agents that restrict iron for erythropoiesis[21–23] and gene editing,[24–28] now tested in clinical trials. Luspatercept (ACE-536), a drug recently approved for TDT[29] and NTDT (https://news.bms.com/news/corporate-financial/2023/), induces sustained elevations of red blood cells (RBC) and hemoglobin (Hb) levels in normal rodents,[30] non-human primates and healthy volunteers,[29–32] as well as in murine models and patients with myelodysplastic syndromes and β-thalassemia.[30,32–34] It is composed of the modified extracellular domain of human activin receptor type IIB fused to the human IgG1 Fc domain, which stimulates erythroid differentiation sequestering TGF ligands, mainly growth differentiation factor 11 (GDF11), *in vitro*.[30,34,35] However, the involvement of GDF11 in the Luspatercept-mediated induction of erythropoiesis *in vivo* has been recently disputed.[36–38] Recent results propose that Luspatercept, by inhibiting the activation of the son-of-mothers against decapenthaplegic 2/3 (SMAD2/3) pathway,[39] would favor the nuclear localization of the transcription intermediary factor 1γ (TIF1γ), thus up-regulating *Gata1* and its erythroid gene signature.[40–42] In this way, the drug would promote erythroid differentiation, without increasing proliferation of early erythroid precursors. Also, Luspatercept murine analogues were able to increase erythropoietin (*Epo*) expression in an NTDT murine model,[43,44] but not in a different one.[30] So, the exact mechanism of the Luspatercept-mediated induction of erythroid differentiation remains unclear.

Transferrin Receptor 2 (TFR2) is a transmembrane protein highly homologous to the iron importer TFR1, mutated in type 3 hereditary hemochromatosis.[45] As TFR1, also TFR2 binds iron-loaded transferrin (holo-TF), even if with a lower affinity and a regulatory, rather than an iron import, function.[46,47] In iron-replete conditions, the binding to holo-TF stabilizes TFR2 on the plasma membrane by inhibiting its proteolytic cleavage[48] and reducing its lysosomal degradation.[49] TFR2 is expressed in the liver and, to a lower extent, in erythroid cells.[50] While the involvement of TFR2 in the regulation of hepcidin has been extensively studied,[45,51] the erythroid function of the protein remained elusive for years, until it was demonstrated that TFR2 and the erythropoietin receptor (EPOR) are synchronously activated and co-expressed during erythroid differentiation.[52] Interestingly, mice with hematopoietic-specific *Tfr2* deletion ($Tfr2^{BMKO}$) show enhanced erythroid differentiation in the presence of comparable EPO levels,[53,54] accompanied by an increase in RBC count and hemoglobin levels, mainly in conditions of high erythroid demand.[54] The mechanism responsible for the TFR2-mediated modulation of erythropoiesis is not completely understood, but *Tfr2*-deficient erythroid cells are more sensitive to EPO stimulation.[54] Overall, these findings identify TFR2 as an important negative regulator of erythropoiesis, that adjusts RBC production according to the available iron. Moreover, hematopoietic *Tfr2* deletion ameliorates IE, anemia, and iron parameters in a murine model of NTD β-thalassemia,[53] and abolishes blood transfusion requirement for survival in TD mice.[55] These results indicate that TFR2 targeting might be a promising therapeutic option for the disease.

We hypothesized that hematopoietic *Tfr2* deletion, enhancing EPO signaling, could improve the therapeutic efficacy of Luspatercept. Here we demonstrate that the murine analog RAP-536 has an additive effect to hematopoietic *Tfr2* deletion in ameliorating anemia and IE in NTDT $Hbb^{th3/+}$ mice, and that RAP-536 activity is independent from the EPO-pathway. In addition, hematopoietic *Tfr2* deletion, but not RAP-536 alone, attenuates iron overload and hepatic inflammation. Taken together our results suggest that combining Luspatercept with hematopoietic *TFR2* targeting might become a promising therapeutic approach to significantly improve the outcome of β-thalassemia patients.

2 | METHODS

2.1 | Animal models

$Hbb^{th3/+}$ mice (with heterozygous deletion of b1 and b2 genes) on a pure C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). *Tfr2-ko* mice on a pure 129S2 background were generated as described.[56,57] $Hbb^{th3/+}$ and *Tfr2-ko* mice were crossed to generate *Tfr2-ko*/$Hbb^{th3/+}$ and $Hbb^{th3/+}$ progenies on a mixed C57/129S2 background.

Bone marrow (BM) cells isolated from 12-week-old C57/129S2 *Tfr2-ko*/$Hbb^{th3/+}$ and $Hbb^{th3/+}$ male mice were used for BM transplantation (BMT), to generate thalassemic mice with ($Hbb^{th3/+}$) or without *Tfr2* ($Tfr2^{BMKO}$/ $Hbb^{th3/+}$) in the hematopoietic compartment. In brief, 5×10^6 BM cells were recovered from the femur of *Tfr2-ko*/$Hbb^{th3/+}$ double mutants and $Hbb^{th3/+}$ mice (expressing the CD45.2 surface antigen) and transplanted, trough retro-orbital injection, into lethally irradiated C57BL/6-Ly-5.1 male mice (expressing the CD45.1 surface antigen). Animals were fed a standard diet and maintained in the animal facility of San Raffaele Scientific Institute in accordance with the European Union guidelines. The study was approved by the Institutional Animal Care and Use Committee of the Institute.

2.2 | Experimental protocol

Nine weeks after BMT, $Hbb^{th3/+}$ and $Tfr2^{BMKO}$/$Hbb^{th3/+}$ mice were subcutaneously treated with RAP-536 (10 mg/kg), the murine analog

of Luspatercept (Celgene, BMS group), or vehicle, twice a week for 8 weeks. A blood sample for complete blood count (CBC) was recovered by tail vein puncture every 2 weeks starting from the beginning of the treatment. At sacrifice, blood and organs were collected for complete phenotypic characterization.

2.3 | Hematological analysis

CBC was measured on an IDEXX Procyte dx automated blood cell analyzer (Idexx Laboratories).

Transferrin saturation was calculated as the ratio between serum iron and total iron binding capacity, using The Total Iron Binding Capacity Kit (Randox Laboratories Ltd.), according to the manufacturer's instructions. Serum EPO levels were measured using mouse EPO quantikine set (R&D System), according to the manufacturer's instructions.

2.4 | Flow cytometry

For phenotypic analysis of erythroid maturation by flow cytometry, BM and spleen cells were stained with PE-C7 rat anti-mouse CD45, PE-C7 rat anti-mouse B220, FITC rat anti-mouse Ter119 and APC rat anti-mouse CD44 (BD Biosciences) for 30 min in the dark at 4°C as previously reported.[58] Donor/host chimerism was evaluated on BM cells from transplanted mice by using FITC rat anti-mouse CD45.1 and APC rat anti-mouse CD45.2 antibodies (BD Biosciences). Samples were acquired at Gallios/Navios cytometer (Beckman Coulter) and results analyzed by using FCS express software (De Novo Software).

2.5 | Immunofluorescence

Spleen and liver tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin through standard methods and sectioned at a thickness of 4 μm at the Animal Histopathology facility of San Raffaele Hospital. Sections were deparaffinized and then incubated for 1 h in PBS + 0.1% Triton-X + 3% Bovine Serum Albumin, followed by staining with APC rat anti-mouse Ter-119 (BD Biosciences) for 30 min in the dark. Images were acquired with the Sapphire Biomolecular Imager (Azure Biosystems) and mean fluorescence intensity of each section was determined using the Image J software.

2.6 | Histological analysis

Spleen and liver sections prepared as above were stained with Hematoxylin & Eosin by standard methods and digital slide scanning was performed with the ImageScope software at the Animal Histopathology facility of San Raffaele Hospital.

2.7 | Gene expression analysis

RNA was extracted from snap-frozen murine livers, spleens, and kidneys using the guanidinium thiocyanate–phenol–chloroform method (Trifast Reagent, Euroclone), following manufacturer's recommendations. BM RNA was extracted by combining the Trifast Reagent protocol with a resin-based purification (ReliaPrep RNA Miniprep system, Promega), after the addition of isopropanol.

RNA (2 μg) was used for first-strand synthesis of cDNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to manufacturer's instructions. For quantitative polymerase chain reaction (qPCR) analysis, specific murine Assays-on-Demand products (20X) and TaqMan Master Mix (2X) (Applied Biosystems) or specific murine oligos (designed using the NCBI Pick Primer software and generated by Merck) and SYBRgreen Master Mix (2X) (Applied Biosystems) were used, and the reactions were run on CFX96 Real-Time PCR System (BioRad). Each cDNA was normalized to the corresponding level of Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1*) or Ribosomal Protein L13 (*Rpl13*). Primers used for qRT-PCR are listed in Supplementary Tables S1 and S2.

2.8 | Tissue iron content

To measure iron concentration, tissue samples were dried at 65°C for 1 week, weighted, and digested in 1 mL of acid solution (3 M HCl, 0.6 M trichloroacetic acid) for 20 h at 65°C. The clear acid extract was added to 1 mL of 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). The solutions were then incubated for 30 min at room temperature until color development and the absorbance measured at 535 nm. A standard curve was plotted, using an acid solution containing increasing amounts of iron sulfate diluted from a stock solution (Merck).

2.9 | Statistics

Data are presented as mean ± standard deviation. One-way and 2-way ANOVA were performed using GraphPad Prism 8.0 (GraphPad). Selected comparisons were evaluated also through a Student t-test analysis. $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | RAP-536 and hematopoietic *Tfr2* deletion synergistically ameliorate anemia and ineffective erythropoiesis of $Hbb^{th3/+}$ mice

To evaluate the effect of a combinatorial strategy based on hematopoietic *Tfr2* deletion and RAP-536 treatment in a model of NTDT, we generated $Hbb^{th3/+}$ mice with or without *Tfr2* ($Tfr2^{BMKO}/Hbb^{th3/+}$) in

BM-derived cells through BMT. Nine weeks after BMT, mice were treated with RAP-536 (10 mg/kg), twice a week for 8 weeks.

Complete engraftment is confirmed through the evaluation of both BM chimerism (Supplementary Figure S1A) and the evaluation of *Tfr2* expression in BM (Supplementary Figure S1B) and spleen (Supplementary Figure S1C). As previously shown,[53] hematopoietic *Tfr2* deficiency ameliorates anemia (Figure 1A), splenomegaly (Figure 1B, C), and IE (Figure 1D, E) of $Hbb^{th3/+}$ mice, an effect which is maintained at least until 34 weeks after transplantation (Supplementary Figure S2). RAP-536 increases RBC count, Hb levels, and HCT already after 2 weeks of treatment with a comparable efficacy in both $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, and the beneficial effect is maintained for the entire protocol (Figure 1A). No significant changes are observed in MCV, MCH, and reticulocytes in both groups of mice. Splenomegaly and red pulp expansion are improved by hematopoietic *Tfr2* deletion, but not by RAP-536 in $Hbb^{th3/+}$ mice (Figure 1B–D and Supplementary Figure S1D,E). This is accompanied by the amelioration of IE both in BM (Figure 1E) and spleen (Figure 1F), with reduced proportion of immature erythroblasts and increased mature RBCs. Erythroid differentiation is further improved in RAP-536-treated $Tfr2^{BMKO}/Hbb^{th3/+}$ mice both in BM (Figure 1E) and spleen (Figure 1F). Of note, spleen size and architecture are almost comparable to those of wild-type animals (Figure 1B–D and Supplementary Figure S1D,E). Severe anemia and hypoxia in $Hbb^{th3/+}$ mice are associated to compensatory hepatic extramedullary erythropoiesis and *Epo* production in the attempt of sustaining the erythropoietic activity. Indeed, some erythroid foci are visible in the liver of thalassemic animals (Supplementary Figure S1F), together with high positivity to the erythroid marker Ter119/Glycophorin A (*Gypa*) both at protein (Figure 1G,H) and mRNA level (Figure 1I) and remarkable *Epo* expression (Figure 1L). All these features are reduced or even abrogated by hematopoietic *Tfr2* deletion, consistent with hypoxia and hepatic extramedullary erythropoiesis correction, while RAP-536 has a very modest effect.

Overall, these data prove that hematopoietic *Tfr2* genetic inactivation acts synergistically with Luspatercept in ameliorating anemia, and ineffective and extramedullary erythropoiesis in the $Hbb^{th3/+}$ murine model of NTD β-thalassemia.

3.2 | RAP-536 does not correct iron overload in both $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice

Since iron overload plays an important role in the development of complications in β-thalassemia, we analyzed iron parameters both at serum and tissue levels. As previously shown, $Tfr2^{BMKO}/Hbb^{th3/+}$ mice are characterized by a trend toward a reduction in serum iron (Figure 2A) and transferrin saturation (TS, Figure 2B), with no changes in total transferrin levels (Total Iron Binding Capacity, TIBC, Figure 2C). Liver (LIC, Figure 2D), heart (HIC, Figure 2E), and kidney iron content (KIC, Figure 2F) are decreased in $Tfr2^{BMKO}/Hbb^{th3/+}$ relative to $Hbb^{th3/+}$ mice. RAP-536 treatment prevents HIC reduction in $Tfr2^{BMKO}/Hbb^{th3/+}$ mice but does not modify iron levels in serum or in other tissues in both groups of mice, excluding a direct role for RAP-536 in the regulation of systemic iron homeostasis, according to previous findings.[14] Of note, iron content of erythropoietic organ, such as spleen (SIC), shows a trend toward reduction in RAP-treated mice (Figure 2G), in accordance with the partial amelioration of IE. Interestingly, mice with maximal IE improvement, namely the RAP-536 treated $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, show the lowest SIC among all groups of animals.

In line with lower LIC, the expression of the iron-sensitive Bone Morphogenetic Protein 6 (*Bmp6*) is reduced in $Tfr2^{BMKO}/Hbb^{th3/+}$ mice as compared to $Hbb^{th3/+}$ (Figure 2H), while *Bmp2* is unchanged (Figure 2I), leading to the downregulation of the BMP–SMAD pathway and decreased mRNA levels of the target genes *Hamp* (Figure 2L) and Inhibitor of Differentiation 1 (*Id1*, Figure 2M). However, *Hamp* mRNA levels appears more appropriate to the systemic iron loading (*Hamp*/LIC ratio, Figure 2N) in $Tfr2^{BMKO}/Hbb^{th3/+}$ mice as compared to $Hbb^{th3/+}$, likely because of the reduction of inhibitory signals (i.e., the erythroid regulator Erythroferrone and others) from the more effective erythropoiesis. On the contrary, RAP-536 does not influence the expression of *Bmp6*, *Bmp2*, *Hamp* and *Id1*, confirming the lack of effect on the regulation of systemic iron homeostasis.

3.3 | Hematopoietic *Tfr2* deletion limits inflammation in $Hbb^{th3/+}$ mice

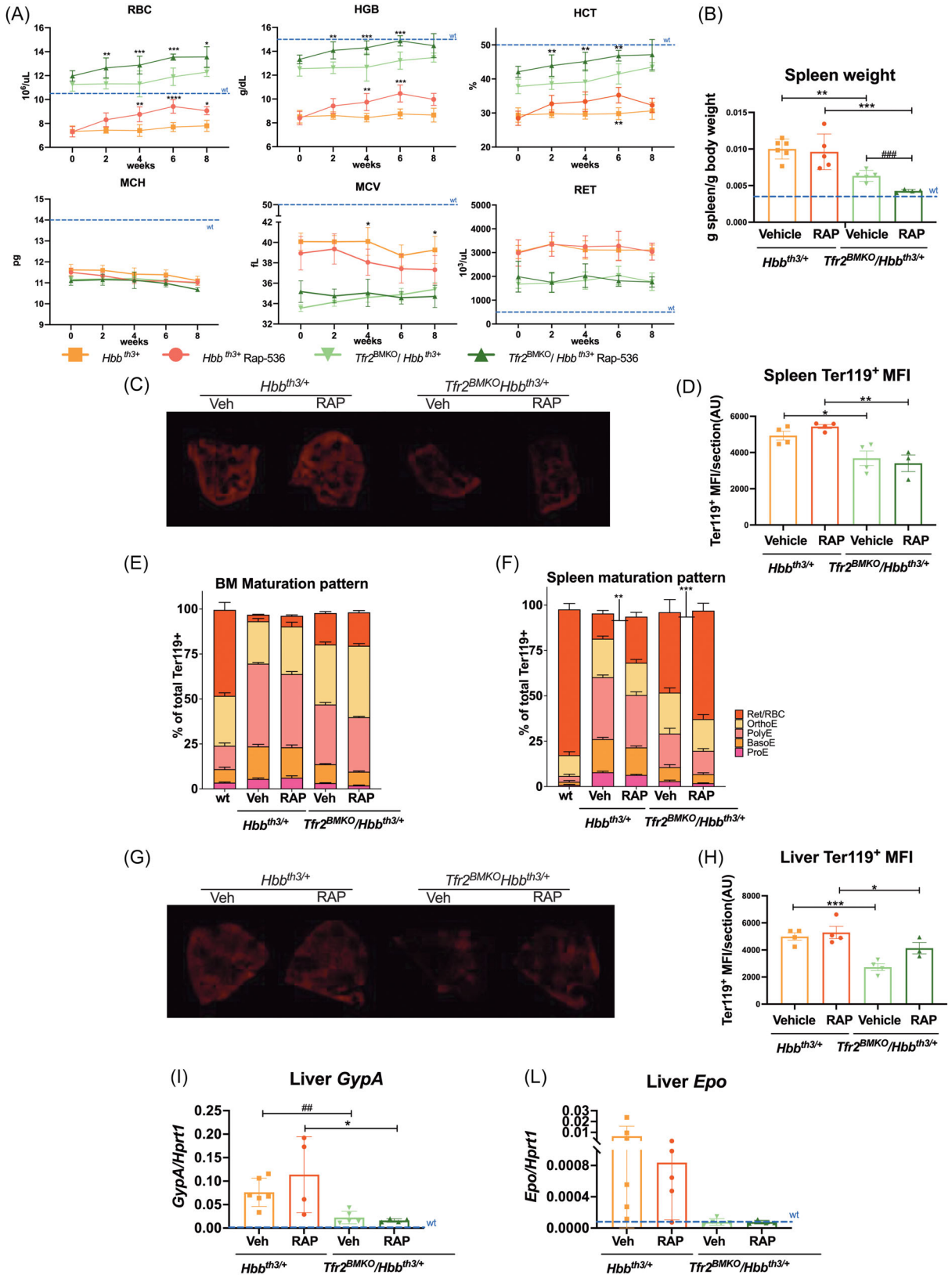
In β-thalassemia iron overload is associated with severe oxidative stress and inflammation, mediators of cell and organ injury.[59] Therefore we investigated the effect of hematopoietic *Tfr2* deletion and RAP-536 on the pro-inflammatory status of $Hbb^{th3/+}$ mice. $Tfr2^{BMKO}/Hbb^{th3/+}$ animals show decreased hepatic expression of *Cd45* (Figure 2O), a marker of leucocyte infiltration, of the acute phase protein α-2-macroglobulin (*A2m*, Figure 2P) and of the STAT3 target genes Tumor Growth Factor β1 (*Tgf-β1* Supplementary Figure S3A) and B-Cell Lymphoma 2 (*Bcl2*, Supplementary Figure S3B) relative to $Hbb^{th3/+}$ mice, suggesting reduced systemic inflammation. In agreement, mRNA levels of the pro-inflammatory cytokine interleukin-6 (*Il-6*) and interleukin-1β (*Il-1β*) are decreased in the liver of mice lacking *Tfr2* in BM-derived cells (Supplementary Figure S3C,D). A similar trend is observed for *Il-6* levels in BM (Supplementary Figure S3E), but not in the spleen (Supplementary Figure S3F). RAP-536 does not affect *Cd45*, *Il6* and *Il1β* levels in both $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, and only mildly decreases the transcription of *A2m* in the liver. However, no differences among the genotypes and treatments were observed in circulating total white blood cells and relative subpopulations (Supplementary Figure S4).

Overall, our findings suggest that the addition of hematopoietic *Tfr2* deletion to Luspatercept, besides correcting anemia more efficiently, improves the main disease complications, such as iron-overload and inflammation.

3.4 | EPO is inappropriately high in RAP-536 treated mice

As expected, anemia amelioration leads to a strong downregulation of serum EPO levels in $Tfr2^{BMKO}/Hbb^{th3/+}$ mice (Figure 3A) and of its

expression in the kidney (Figure 3B). Interestingly, this does not occur upon RAP-536 treatment in both *Hbb*$^{th3/+}$ and *Tfr2*^{BMKO}/*Hbb*$^{th3/+}$ mice, in which, despite higher hemoglobin, *Epo* mRNA and protein levels are comparable, or even higher than those of vehicle-treated animals of the same genotype. This suggests that RAP-536 treatment might directly increase kidney *Epo* expression, in line with previous



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results obtained with its analog RAP-011.[43] We hypothesized that this effect might be mediated by the inhibition of the TGF-β signaling, since a recent study showed that TGF-β decreases kidney *Epo* production repressing the transcription of *Epas1* (the gene encoding for the *Epo* activating protein HIF-2α) in EPO-producing cells.[60] However, *Epas1* mRNA levels in the kidney are not affected by RAP treatment (Figure 3C), excluding that this mechanism is involved in the maintenance of high EPO levels.

Low EPO limits the proliferation of early erythroid precursors, further contributing to the correction of IE and restricting the production of the erythroid regulator erythroferrone (*Erfe*) in *Tfr2*^{BMKO}/*Hbb*$^{th3/+}$ animals, both in BM (Figure 3D) and spleen (Figure 3E), resulting in reduced circulating levels of the hormone (Figure 3F). Since ERFE is a negative regulator of hepcidin expression,[61] its reduction is possibly responsible for the more appropriately high hepcidin levels of double mutant mice. No changes in BM and spleen *Erfe* levels are observed upon RAP-536 treatment in both genotypes, likely because of the sustained EPO production. Consistently, the expression of the EPO-EPOR target genes *Bcl-xl* (Supplementary Figure S5A,B) and *Epor* itself (Supplementary Figure S5C,D) is not affected by RAP-536 treatment, further supporting the hypothesis of its EPO-independent effect. On the contrary, mRNA levels of *Bcl-xl* and *Epor* of *Tfr2*^{BMKO}/*Hbb*$^{th3/+}$ animals, despite comparable to values measured in *Hbb*$^{th3/+}$, are inappropriately high relative to low EPO (Figure 3G–L). Similar results are observed for additional EPO-EPOR target genes, such as Cyclin G2 (*Ccng2*) and cKit, activated by the EPO-AKT pathway, and TLC domain containing 4 (*Tlcd4*) and Cellular Inhibitor of Apoptosis (*cIap*), activated by the EPO-ERK signaling (Supplementary Figure S5E-N). The same trend is evident also when *Erfe* levels are normalized on serum EPO (Supplementary Figure S5O-P). Overall, these findings confirm the enhanced EPO responsiveness of *Tfr2*-deficient erythroid cells.[53,54]

3.5 | Chronic RAP-536 treatment does not inhibit SMAD2/3 signaling pathway

To better investigate the mechanism through which RAP-536 promotes erythroid differentiation in our model, we evaluated the activation of the SMAD2/3 pathway, which is expected to be downregulated upon Luspatercept treatment. Surprisingly, the expression of *cMyc* (Figure 4A,B), *Id1* (Figure 4C,D), *Pai1* (Figure 4E,F), and *Smad7* (Figure 4G,H), target genes positively modulated by SMAD2/3 pathway,[62,63] is unchanged or even increased upon RAP-536 treatment both in BM and spleen, indicating that, in chronic conditions, the TGFβ-SMAD2/3 pathway is not inhibited by Luspatercept. Activin Ligand Traps have been proposed to promote differentiation enhancing apoptosis of defective immature erythroblasts through the FAS–FASL pathway.[43] In line with this observation, *Fasl* mRNA is augmented in the BM of *Hbb*$^{th3/+}$ treated mice (Figure 4I) and show a trend toward an increase in the spleen of RAP-treated *Tfr2*^{BMKO}/*Hbb*$^{th3/+}$ animals (Figure 4L), suggesting a pro-apoptotic phenotype.

4 | DISCUSSION

Despite the recent approval of innovative approaches, such as gene therapy[16] and Luspatercept,[29,33,43] the treatment of β-thalassemia, a disease with about 60.000 affected individuals born every year, is still far from optimal. This is likely due to the complex pathophysiology of the disorder, with several contributing and modifier factors, in addition to the genetic defect. In agreement, combinatorial treatments targeting different pathologic cues of the disease have been shown to be more effective than single agent strategies in preclinical studies.[22] Here, we demonstrate that RAP-536, the murine orthologue of Luspatercept that promotes the maturation of late-stage erythroblasts,[40] and hematopoietic *Tfr2* deletion, that increases EPO-sensitivity of early erythroid precursors,[54] synergize in the correction of anemia and IE in *Hbb*$^{th3/+}$ mice, proving that the two approaches act through different mechanisms. Similar findings have been obtained combining an analog activin-ligand trap with agents that redistribute iron, as anti *Tmprss6* antisense oligonucleotides (ASO) (Guerra A. et al., submitted) or monoclonal antibodies,[64] further supporting the therapeutic potential of combination strategies for the treatment of β-thalassemia.

Hematopoietic *Tfr2* inactivation improves anemia and IE of thalassemic mice more than RAP-536, and the combined approach has an impressive effect, raising RBC count and Hb levels to wild-type values. An additive effect of the two strategies is observed also in the improvement of IE both in the BM and, even more strikingly, in the spleen, leading to an almost complete correction of splenomegaly

FIGURE 1 Additive therapeutic effect of RAP-536 and hematopoietic *Tfr2* deletion in *Hbb*$^{th3/+}$ mice. *Hbb*$^{th3/+}$ and *Tfr2*^{BMKO}/*Hbb*$^{th3/+}$ mice were generated through bone marrow (BM) transplantation, and 9 weeks later were treated with RAP-536 (RAP, 10 mg/kg, s.c.) or vehicle (Veh) twice a week for 8 weeks. In the figure are reported: (A) red blood cell (RBC) count, hemoglobin (Hb) levels, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and reticulocyte percentage (RET); (B) spleen weight relative to body weight; (C) Ter119 immunofluorescence on deparaffinated spleen slices and (D) relative mean fluorescence quantification (MFI); (E, F) erythroid cells differentiation in the BM and in the spleen evaluated based on the relative expression of CD44 and Ter119, and on cells size. Gated populations: proerythroblasts (I), basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts and immature reticulocytes (IV) and mature red cells (V); (G) Ter119 immunofluorescence on deparaffinated liver slices and (H) relative MFI quantification; hepatic mRNA levels of (I) Glycophorin A (*GypA*) and (L) erythropoietin (*Epo*) relative to the housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt1*). Mean values of 4-6 mice per group are reported. Dotted blue line indicates mean value of wild-type (wt) mice. Error bars represent standard deviation (SD). Asterisks indicate statistically significant differences calculated by Two-way (A) or One-way (B–L) ANOVA. ****$p < .0001$; ***$p < .001$; **$p < 0.01$. Hashtags indicate statistically significant differences by Student *t* tests. ###$p < .001$; ##$p < .01$.

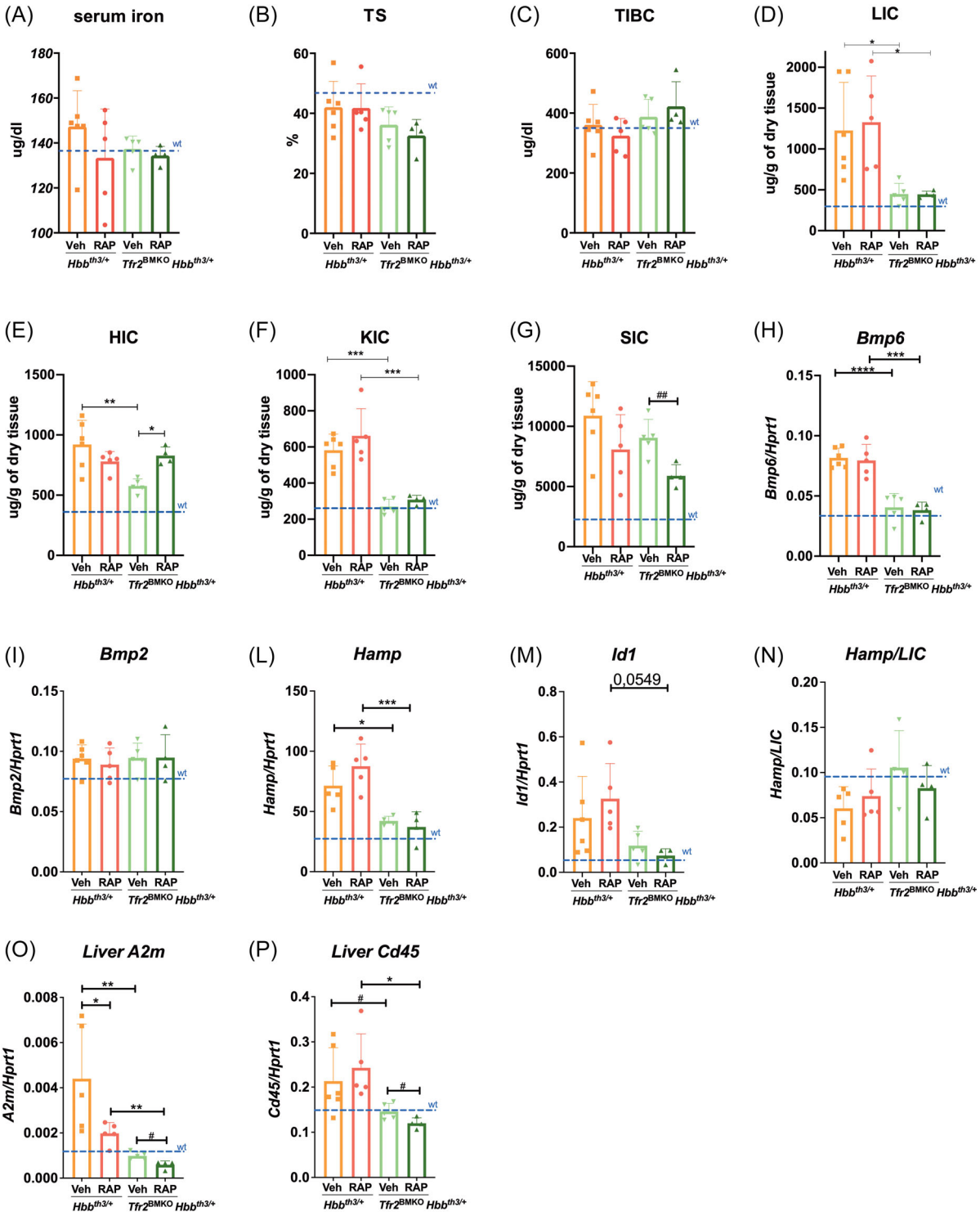

FIGURE 2 Hematopoietic *Tfr2* deletion, but not RAP-536, reduces iron-overload and inflammation of $Hbb^{th3/+}$ mice. $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice were generated through bone marrow (BM) transplantation and sacrificed after 8 weeks of treatment with RAP-536 (10 mg/kg, s.c.) or vehicle (Veh). In the figure are reported: (A) serum iron; (B) Transferrin Saturation (TS); (C) Total Iron Binding Capacity (TIBC); iron content in (D) the liver (LIC), (E) heart (HIC), (F) kidney (KIC) and (G) spleen (SIC); hepatic mRNA levels of (H) Bone Morphogenetic Protein 6 (*Bmp6*), (I) Bone Morphogenetic Protein 2 (*Bmp2*), (L) hepcidin (*Hamp*) and (M) Inhibitor of Differentiation 1 (*Id1*) relative to the housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt1*); (N) the ratio between hepatic *Hamp* levels and LIC (*Hamp*/LIC); hepatic mRNA levels of (O) alpha 2 macroglobulin (*A2m*) and (P) cluster of differentiation 45 (*Cd45*) relative to the housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt1*). Mean values of 4-6 mice per group are reported. Dotted blue line indicates mean value of wild-type (wt) mice. Error bars represent standard deviation (SD). Asterisks indicate statistically significant differences calculated by One-way ANOVA. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$. Hashtags indicate statistically significant calculated by Student *t* tests. ##$p < .01$; #$p < .05$. *p*-values close to .05 are also reported.

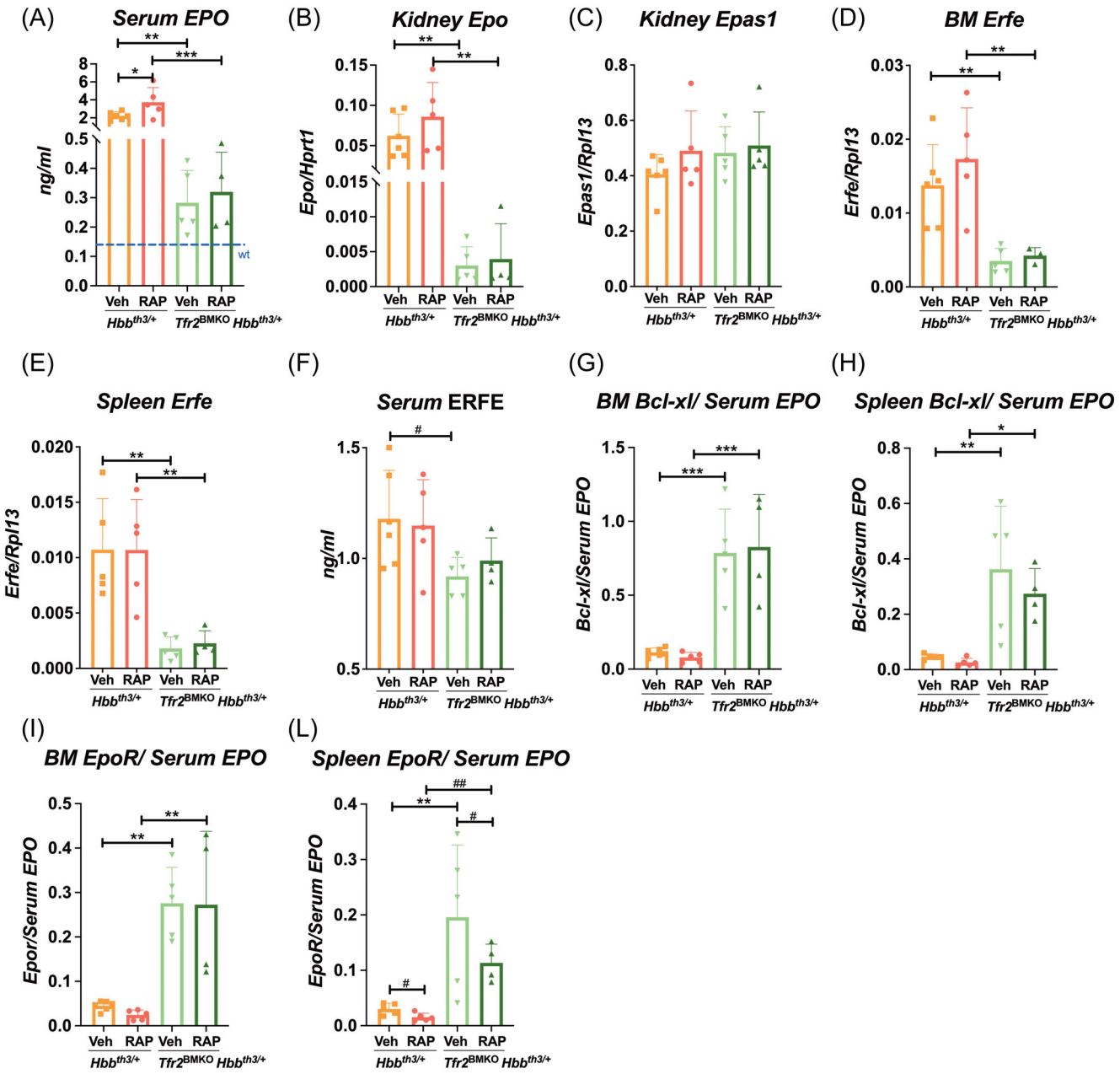

FIGURE 3 RAP-536 does not stimulate erythropoiesis through the EPO-EPOR signaling pathway. $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, obtained through bone marrow (BM) transplantation, were sacrificed after 8 weeks of treatment with RAP-536 (10 mg/kg, s.c.) or vehicle (Veh). In the figure are reported: (A) serum erythropoietin (EPO) levels; mRNA levels of (B) *Epo* and (C) Endothelial PAS Domain Protein 1 (*Epas1*) in the kidney and of (D, E) erythroferrone (*Erfe*) in the BM and spleen; (F) serum ERFE levels; mRNA levels of (G,H) the antiapoptotic factor B-cell lymphoma-extra large (*Bcl-Xl*) and (I, L) EPO receptor (*Epor*) in the BM and in the spleen normalized on serum EPO. Values are expressed relative to the housekeeping gene Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1*) for the kidney and to ribosomal protein L13 (*Rpl13*) for the BM and the spleen. Mean values of 4-6 mice per group are reported. Dotted blue line indicates mean value of wild-type (wt) mice. Error bars represent standard deviation (SD). Asterisks indicate statistically significant differences calculated by One-way ANOVA. ***$p < .001$; **$p < .01$; *$p < .05$. Hashtags indicate statistically significant differences by Student *t* tests. ##$p < .01$; #$p < .05$.

in RAP-treated $Tfr2^{BMKO}/Hbb^{th3/+}$ mice. In addition, the combined treatment abrogated *Epo* production by the liver, a feature associated with severe anemia and hypoxia. Of note, since RAP-536 ameliorates anemia of both $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice with a comparable efficacy, it modulates erythropoiesis independently from TFR2, which acts to enhance EPO signaling in erythroid cells.

Tfr2 is expressed in other cells of hematopoietic origin, such as macrophages, which are replaced by donor cells through BM transplantation procedure. So, in principle, a contribution of *Tfr2*-deficient macrophages to the phenotype of $Tfr2^{BMKO}/Hbb^{th3/+}$ mice cannot be ruled-out. Evaluating strategies alternative to BMT, CRE-Flox mice would not be a solution, since no CRE model is currently available to

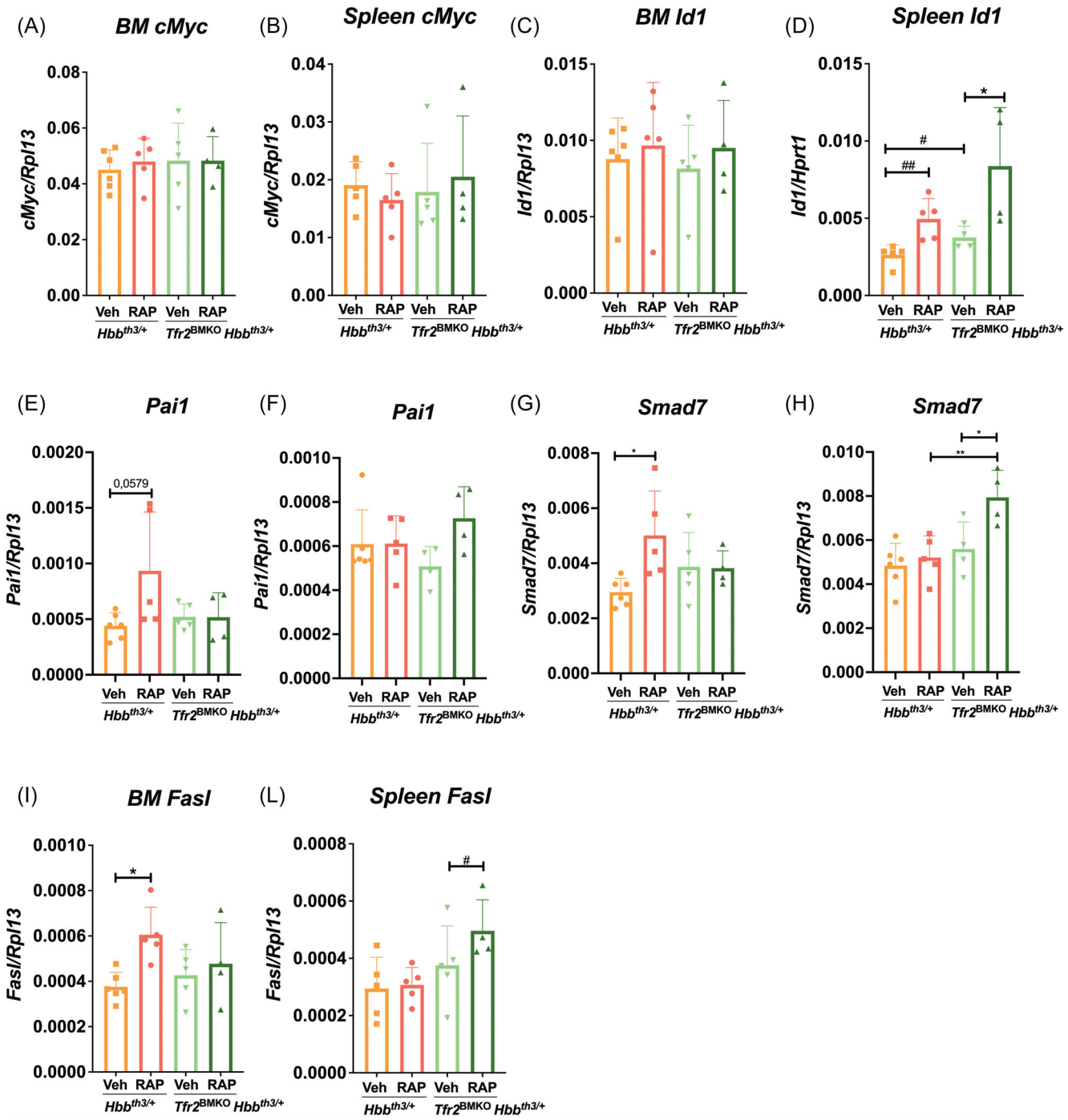

FIGURE 4 RAP-536 treatment does not inhibit the SMAD2/3 pathway in chronic conditions. $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, generated through bone marrow (BM) transplantation, were sacrificed after 8 weeks of treatment with RAP-536 (10 mg/kg, s.c.) or vehicle (Veh). In the figure are reported: mRNA levels of (A, B) the myelocytomatosis oncogene (*cMyc*), (C, D) the inhibitor of differentiation (*Id1*), (E, F) the plasminogen activated inhibitor 1(*Pai1*), (G, H) the small mothers against decapenthaplegic 7 (*Smad7*), and (I, L) the pro-apoptotic gene Fas ligand (*Fasl*) relative to the housekeeping gene ribosomal protein L13 (*Rpl13*) in the BM and in the spleen. Mean values of 4–6 mice per group are reported. Error bars represent standard deviation (SD). Asterisks indicate statistically significant differences calculated by One-way ANOVA. $^{*}p < .05$, $^{**}p < .01$. *p*-values close to .05 are also indicated. Hashtags indicate statistically significant differences calculated by Student t tests. $\#\#p < .01$; $\#p < .05$.

achieve erythroid-specific gene inactivation. As in our model, *Vav*-CRE mice inactivate the gene of interest in all hematopoietic cells, whereas *Epor*-CRE inactivates the gene of interest in all *Epor* expressing cells, which means not only hematopoietic cells[65] but also brain cells,[66] endothelial cells,[67] and cardiomyocytes.[68] Of note, no iron or erythroid phenotype has been described for mice with macrophage-specific *Tfr2* inactivation.[69] Also, the improved inflammatory status of $Tfr2^{BMKO}/Hbb^{th3/+}$ mice is likely macrophage-independent, since

Tfr2-deficient macrophages showed a more pronounced pro-inflammatory behavior in a model of experimental arthritis.[70] Therefore, we concluded that an effect of *Tfr2* deficient macrophages is unlikely in our setting.

The interference of activin ligand traps on EPO function in the modulation of erythropoiesis is a controversial issue. A previous study showed that RAP-536 treatment corrected anemia and EPO production in thalassemic mice.[33] However, RAP-011, the activin ligand trap composed of the extracellular domain of ActRIIA instead of ActRIIB, whose activity is similar to that of RAP536, increased EPO production in thalassemia murine models,[43] claiming for a potential EPO-mediated function of the compound. In our model despite anemia correction, EPO is not reduced, but even increased in RAP-treated mice, a finding that may explain the lack of beneficial effect on splenomegaly. The mechanism responsible for the maintenance of high EPO is still unknown, but likely involves the stabilization of HIF-2α protein since we excluded that RAP-536 increases *Hif-2α* mRNA or modulates iron content in the kidney. However, since RAP-536 has comparable effect in both $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, characterized by different EPO sensitivity, increased EPO production is unlikely to be the main driver of anemia improvement by the drug.

HIF-2α stabilization following RAP-536 treatment might be also responsible for the lack of reduction of heart iron loading. Indeed, in *Tfr2*-deficient mice anemia and hypoxia correction might limit heart hepcidin expression, which is specifically upregulated by hypoxia,[71] thus promoting local iron release. RAP-536, stabilizing HIF, might prevent heart hepcidin downregulation in $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, thus maintaining HIC comparable to vehicle-treated $Hbb^{th3/+}$ animals. However, the drug does not worsen heart iron loading of thalassemic mice carrying *Tfr2*, so this observation is likely not a concern for Luspatercept treatment.

The proposed mechanism of action of activin ligand traps includes the inhibition of the TGFβ-SMAD2/3 signaling pathway and the promotion of FASL-mediated apoptosis of defective erythroblasts.[43] Here, we show that, in chronic conditions (8 weeks of treatment), RAP-536 is unable to reduce the activation of the SMAD2/3 pathway, suggesting that inhibition of this signaling is not crucial to the prolonged activity of the drug. On the contrary, RAP-536 increases *Fasl* expression in erythroid tissues, suggesting that the pro-apoptotic activity of Luspatercept contributes to erythropoiesis improvement in long term. However, the exact mechanism of action of the drug remains to be fully elucidated.

In line with our previous findings,[53,54] hematopoietic *Tfr2* deficiency significantly improves iron parameters of thalassemic mice, likely because of the more effective erythropoiesis and low EPO levels, which reduce the production of the erythroid regulator *Erfe*. In agreement, *Hamp* relative to LIC is more appropriate, leading to a condition of iron restriction, known to be beneficial to thalassemic erythropoiesis. On the contrary, RAP-536 treatment does not correct iron overload in both $Hbb^{th3/+}$ and $Tfr2^{BMKO}/Hbb^{th3/+}$ mice, with the sole exception of a mild SIC reduction, likely because of diminished erythrophagocytosis, in line with patients' data.[72,73] In addition, the expression of iron-regulatory genes is unchanged in RAP-treated thalassemic animals, consistent with previous findings.[30] The inability of the drug to correct iron overload is likely due to the maintenance of high EPO production despite anemia correction, which keeps ERFE expression high, and unchanged hepcidin and iron.

In this context, combining hematopoietic *Tfr2* deletion with Luspatercept might not only improve the therapeutic efficacy of the drug on erythroid differentiation but also reduce iron overload, thus preventing liver damage. Of note, the partial correction of tissue iron loading driven by *Tfr2* loss reduces hepatic and systemic inflammation in both RAP- and vehicle-treated $Tfr2^{BMKO}/Hbb^{th3/+}$ mice. Thus, our proposed combinatorial approach might also counteract severe complications due to systemic inflammation, among which hypercoagulation and increased hemolysis of fragile thalassemic RBCs,[59,74,75] further contributing to improve anemia.

Several strategies might be envisaged to selectively inhibit *Tfr2* in cells of hematopoietic origin. We recently tested an RNA-interference-based approach. However, unfunctionalized antisense oligonucleotides were unable to efficiently downregulate *Tfr2* mRNA in erythroid tissues,[76] calling for the identification of targeting moieties, in analogy with the GalNac conjugation for hepatic delivery.[77–79] Gene editing might be an intriguing alternative, likely exploiting recently developed strategies for *in vivo* editing.[80] Further studies are required to identify the most appropriate strategy to translate hematopoietic *Tfr2* deletion into a therapeutic tool.

Overall, our preclinical results suggest that the combination of Luspatercept with approaches that inhibit erythroid *Tfr2* might represent a future option for treatment of β-thalassemic patients, improving anemia, IE, splenomegaly and iron-overload and decreasing the risk of associated complications, such as organ damage and inflammation.

AUTHOR CONTRIBUTIONS

Emanuele Tanzi performed research, analyzed data, and wrote the paper; Simona Maria Di Modica, Jessica Bordini, Violante Olivari, Alessia Pagani and Valeria Furiosi performed experiments; Laura Silvestri and Alessandro Campanella contributed to critical data analysis and paper editing; 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 STATEMENT

The other authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

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

ORCID

Jessica Bordini https://orcid.org/0000-0002-2669-6953
Laura Silvestri https://orcid.org/0000-0002-8726-9808
Alessandro Campanella https://orcid.org/0000-0002-3463-1199
Antonella Nai https://orcid.org/0000-0002-0739-5282

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

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