REGULAR ARTICLE

blood advances

A human anti-matriptase-2 antibody limits iron overload, α-globin aggregates, and splenomegaly in β-thalassemic mice

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Key Points

- RLYB331 is a human antibody that increases hepcidin, reduces iron overload, and ameliorates ineffective erythropoiesis in β-thalassemia
- Combination with RAP-536L, simultaneously corrects iron overload, ineffective erythropoiesis, splenomegaly, and hematological parameters

Iron plays a major role in the deterioration of β-thalassemia. Indeed, the high levels of transferrin saturation and iron delivered to erythroid progenitors are associated with production of α-globin precipitates that negatively affect erythropoiesis. Matriptase-2/TMPRSS6, a membrane-bound serine protease expressed in hepatocytes, negatively modulates hepcidin production and thus is a key target to prevent iron overload in β-thalassemia. To address safety concerns raised by the suppression of *Tmprss6* by antisense oligonucleotides or small interfering RNA, we tested a fully human anti-matriptase-2 antibody, RLYB331, which blocks the protease activity of matriptase-2. When administered weekly to $Hbb^{th3/+}$ mice, RLYB331 induced hepcidin expression, reduced iron loading, prevented the formation of toxic α-chain/heme aggregates, reduced ros oxygen species formation, and improved reticulocytosis and splenomegaly. To increase the effectiveness of RLYB331 in β-thalassemia treatment even further, we administered RLYB331 in combination with RAP-536L, a ligand-trapping protein that contains the extracellular domain of activin receptor type IIB and alleviates anemia by promoting differentiation of late-stage erythroid precursors. RAP-536L alone did not prevent iron overload but significantly reduced apoptosis in the erythroid populations of the bone marrow, normalized red blood cell counts, and improved hemoglobin and hematocrit levels. Interestingly, the association of RLYB331 with RAP-536L entirely reversed the β-thalassemia phenotype in $Hbb^{th3/+}$ mice and simultaneously corrected iron overload, ineffective erythropoiesis, splenomegaly, and hematological parameters, suggesting that a multifunctional molecule consisting of the fusion of RLYB331 with luspatercept (human version of RAP-536L) would allow administration of a single medication addressing simultaneously the different pathophysiological aspects of β-thalassemia.

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The full-text version of this article contains a data supplement.

Introduction

β-thalassemia is a hereditary disorder caused by mutations in the β-globin gene that lead to defective or complete lack of production of β-globin chains of hemoglobin. The resulting excess of free α-globin leads to precipitates that adhere to the cell membrane, leading to oxidative stress, red blood cell (RBC) membrane damage, premature death of late-stage erythroid precursors, and results in ineffective erythropoiesis, that is, overproduction of erythroid progenitors that fail to generate mature erythrocytes. Patients display considerable clinical heterogeneity. Those with β-thalassemia major produce no or very little β-globin, require chronic RBC transfusions to survive, and therefore, develop extreme iron overload secondary to the transfusions. However, even when transfusions are not required as in patients with β-thalassemia intermedia, the manifestations of the disease can be severe, including chronic anemia and splenomegaly due to extramedullary hematopoiesis and increased turnover of damaged RBCs. Anemia and ineffective erythropoiesis also cause increased intestinal iron absorption mediated by duodenal hypoxia and Erythroferrone-mediated suppression of the iron regulatory hormone hepcidin.[1] The resulting iron overload adds to morbidity by causing cirrhosis and cardiomyopathy, and by exacerbating erythroid cell damage, apoptosis, and ineffective erythropoiesis.[2,3] The only currently approved therapy for patients with non–transfusion-dependent β-thalassemia (NTDT), iron chelation, is suboptimal and does not specifically address the underlying pathological mechanisms.[4] In the last decade, new agents have been designed to ameliorate ineffective erythropoiesis (activin receptor II ligand traps[5-7]) or to prevent iron overload (Tmprss6 antisense oligonucleotides or small interfering RNA,[8,9] minihepcidins,[10] ferroportin inhibitors[11]). Although these new agents were all shown to be beneficial in the treatment of β-thalassemia in the mouse, they address each only single pathophysiological aspects of the disease and do not entirely correct the inefficiency of the erythropoiesis and its follow-on effect, suggesting that approaches combining 2 agents with complementary actions should be considered in the future.[4,12]

Matriptase-2/TMPRSS6, a membrane-bound serine protease expressed in hepatocytes, negatively modulates hepcidin production and thus is a key target to prevent iron overload in β-thalassemia.[13] As expected, antisense oligonucleotides[8] or small interfering RNA[9,14] that downregulate TMPRSS6, stimulate hepcidin expression, reduce iron burden, and improve RBC survival in β-thalassemia mouse models. This prompted Ionis Pharmaceuticals to generate a ligand-conjugated antisense medicine, TMPRSS6-L, to treat anemia and iron toxicity in patients with β-thalassemia.[15] This drug is now being evaluated in a phase 2 trial in adults with NTDT and baseline hemoglobin ≤10 g/dL. However antisense oligonucleotides have been found to produce severe adverse effects, such as thrombocytopenia, during the course of several human clinical trials, raising some safety concerns on their long-term use.[16] To provide an alternative treatment paradigm while taking advantage of the beneficial effects of TMPRSS6 downregulation, a first-in-class, fully human monoclonal antibody neutralizing matriptase-2 was developed from transgenic mice harboring the entire human immunoglobulin variable-gene repertoire.[17] In this study, we examine the effects of this new antibody on the β-thalassemia intermedia phenotype of mice with heterozygous deletion of the β-minor and β-major hemoglobin genes ($Hbb^{th3/+}$). These mice have moderately severe, transfusion-independent, hypochromic microcytic anemia associated with ineffective erythropoiesis resulting in splenomegaly, hepcidin suppression, and secondary iron overload.

Because combinatorial approaches with agents whose mechanisms of action are different but complementary, may prove to be superior to single treatments, we also administered the matriptase-2 neutralizing antibody in combination with a RAP-536 like molecule (RAP-536L), the murine version of luspatercept,[18] and tested the benefits of using this drug combination for $Hbb^{th3/+}$ mice. Ligands of the transforming growth factor β (TGF-β) superfamily play an inhibitory role in terminal erythroid differentiation by a Smad2/3-dependent process. Luspatercept is a ligand-trapping protein containing the extracellular domain of activin receptor type IIB modified to reduce activin binding and fused to the Fc domain of immunoglobulin G (IgG) 1. It sequesters ligands of the TGF-β superfamily, particularly GDF8 and GDF11, before they can interact with the receptor, thus inhibiting the Smad2/3 signal transduction cascade. Its murine version, RAP-536, was shown to enhance late-stage erythropoiesis in β-thalassemic mice,[7] possibly by increasing Gata-1 availability in the nucleus.[19] Luspatercept showed encouraging data in a phase 2 study, including improvement in hemoglobin level in NTDT and reduction in transfusion burden in transfusion-dependent β-thalassemia[15] and is now marketed under the name Reblozyl.

We also administered the matriptase-2 neutralizing antibody in combination with darbepoetin alpha because agents enhancing erythropoiesis could increase the production of RBCs and could have a synergic effect with the inhibition of matriptase-2.

Our results indicate that, although RLYB331 alone prevents iron overload, ameliorates ineffective erythropoiesis, and limits the formation of toxic α-chain, it does not correct anemia. On the other hand, RAP-536L alone efficiently corrects anemia but it neither prevents iron overload, nor ameliorates ineffective erythropoiesis to the same extent as RLYB331. The association of the 2 treatments is effective on both iron overload and anemia. To some extent, although less efficient, we obtained similar results with the darbepoetin alpha cotreatment; thus confirming that, combinatorial treatments may be superior to approaches targeting single pathophysiological aspects. Overall, our study suggests that the clinical use of our newly developed, fully human, antibody-neutralizing matriptase-2 in combination with luspatercept, may represent a superior option for a more effective management of β-thalassemia.

Methods

Matriptase-2 neutralizing antibody RLYB331

Transgenic mice genetically engineered to contain the entire human immunoglobulin heavy and light chain variable-gene repertoire[17] were immunized with purified recombinant human and mouse matriptase-2 extracellular domains, full-length human matriptase-2 expressed on cells, and matriptase-2 encoding DNA vectors, generating a diverse range of highly specific monoclonal antibodies. RLYB331 was identified after a multistep screening cascade on the Kymab IntelliSelect platform as a cross-reactive antibody (human/mouse) blocking the protease activity of

matriptase-2 by binding to the serine protease active site. It was shown to increase Bmp/Smad signaling and hepcidin expression in C57Bl/6 mice, following a single IP dose of 10 mg/kg.[20] For long-term assessment in *Hbb*$^{th3/+}$ mice, RLYB331 was reformatted into a mouse IgG1 chimeric antibody to reduce the formation of an antidrug-antibodies response.

Animal studies

β-thalassemic (*Hbb*$^{th3/+}$) mice and wild-type (WT) controls used in this study were all 8-week-old males on a C57BL/6N background. Mice were housed in a constant light-dark cycle. They were given free access to tap water and standard laboratory mouse diet containing 180 parts per million iron (SSNIFF, Soest, Germany). *Hbb*$^{th3/+}$ mice were treated with intraperitoneal injections (10 mg/kg) of the anti-matriptase-2 antibody RLYB331 or a nonrelevant mouse IgG1 isotype control once a week for 8 weeks. RAP-536L, a modified activin receptor type IIB extracellular domain linked to the murine IgG2a-Fc domain biochemically identical to RAP-536,[7] was administered by intraperitoneal injections at the dose of 10 mg/kg twice weekly for 8 weeks. Mice received intraperitoneal injections (30 mg/kg) of darbepoetin alpha (Arasnep, Amgan) once a week. The mice were euthanized 3 days after the last injection. Experimental protocols were approved by the Midi-Pyrénées Animal Ethics Committee and the French Ministry.

Gene expression analysis

Total RNA from mouse liver was extracted using UPzol lysis reagent (biotechrabbit, Hennigsdorf, Germany). Complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega). Quantitative polymerase chain reactions with *Hamp* and *Hprt* primers listed in Belot et al[21] were prepared with LightCycler 480 DNA SYBR Green I Master reaction mix (Roche Diagnostics) and run on a LightCycler 480 System (Roche Diagnostics). ΔCt values were obtained by subtracting the reference gene Ct to the target gene Ct.

Serum hepcidin assay

Serum hepcidin was quantified using the Hepcidin Murine Compte enzyme-linked immunosorbent assay kit (Intrinsic Lifesciences, La Jolla, CA) according to the manufacturer's instructions.

Hematological and iron parameters

Blood was collected after euthanization from the abdominal aorta in EDTA-coated tubes. Hematological parameters were assessed on a CELL-DYN Emerald system (Abbott, Lake Forest, IL). Serum iron concentration was measured by the Anexplo core facility of Toulouse. The Reticulocyte Reagent System (BD 349204) was used to determine reticulocytes as a percentage of total erythrocytes in the peripheral blood.

Tissue iron measurements and iron staining

Quantitative measurement of nonheme iron in the liver was performed according to the method recommended by Torrance & Bothwell.[22] Liver samples were fixed in 4% buffered formalin and embedded in paraffin. Deparaffinized tissue sections were stained with the Perls' Prussian blue stain for nonheme iron and counterstained with nuclear fast red. Slides were scanned on a Pannoramic 250 Flash II (3DHISTECH) and analyzed with the Pannoramic Viewer software.

Erythroid differentiation in bone marrow and spleen

Erythroid cells were analyzed from the spleen and the bone marrow by flow cytometry. Cell suspensions were stained in phosphate-buffered saline (PBS) supplemented with sterile 5% fetal bovine serum (FBS). The following monoclonal antibodies were used for murine flow cytometric analysis: anti-Ter119-PE (BD Biosciences, 553673), anti-CD44-PE-Cy7 (BD Biosciences, 560599). The lineage (Lin) APC-conjugated antibodies were used: anti-Ly-6G (BD Biosciences, 560599), anti-Cd11c (BD Biosciences, 561119), anti-CD3 (BD Biosciences, 565643), anti-cd11b (BD Biosciences, 553312), anti-CD49b (BD Biosciences, 560628), anti-CD19 (BD Biosciences, 550992). Specifically, erythrocytes were defined as Lin$^-$, Ter119$^+$. This assay allows the separation of erythroid cells into distinct populations corresponding to (I) proerythroblasts, (II) basophilic, (III) polychromatic, (IV) orthochromatic cells and reticulocytes, and (V) RBCs (supplemental Figure 1). A minimum of 250 000 events were recorded for erythrocytes in spleen and bone marrow. For all the analyses, cells were acquired using the MACSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec) and the results analyzed with FlowJo software (Tree Star Inc, Ashland, OR).

Detection of membrane α-globins in erythroid cells

Blood samples were collected and membrane cytoskeletons were prepared as previously described[23] from the same number of erythrocytes for each sample (150 × 10^6). Briefly, the erythrocyte ghosts were washed 3 times by resuspending in hypotonic lysis buffer and centrifugation at 21 000*g*. Membrane lipids were extracted in 50 mM sodium borate pH 8, 1mM EDTA, 0.5% Triton X-100 (Sigma-Aldrich), and protease inhibitors. After a last 30 minutes centrifugation at 30 000*g* the supernatant was completely removed and the triton-insoluble pellet corresponding to the membrane cytoskeletons was snap frozen and analyzed using Triton/acetic acid/urea polyacrylamide gel electrophoresis, as previously described.[24]

Reactive oxygen species measurement

Cells from the spleen and bone marrow were washed and resuspended in PBS supplemented with 5% FBS and incubated with 5-(and −6)-choloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) stain (Invitrogen, C6827) (5 μmol/L) in the dark for 15 minutes at 37°C, centrifugated 5 minutes at 300*g*, and resuspended in PBS 5% FBS. The oxidative conversion of CM-H_2DCFDA to its fluorescent product by ros oxygen species (ROS) was measured immediately by flow cytometry. The ROS fluorescence signals (median fluorescence intensity) were recorded in the Lin$^-$ Ter119$^+$ cell population. For all the analyses, cells were acquired using the MACSQuant Analyzer 10 Flow Cytometer and the results analyzed with FlowJo software (Tree Star Inc).

Apoptosis assessment

Apoptosis in erythroid precursors was determined by flow cytometry using an apoptosis detection kit (BD Biosciences). Erythroid precursors in spleen and bone marrow were identified as previously described and counterstained with annexin V labeled with Pacific blue (Biolegend, 640918). The percentages of annexin V$^+$ among Ter-119$^+$ cells were gated and the data recorded.

Statistical analysis

Experimental data are presented as Box plots or stacked bar charts. One-way analysis of variance with Brown-Forsythe and Welch tests, followed by Tamhane's T2 multiple comparisons tests, was performed using GraphPad Prism Version 9.3.0 (GraphPad Software Inc, San Diego, CA).

Results

The anti-matriptase-2 monoclonal antibody RLYB331 induces hepcidin expression and reduces iron overload in Hbb$^{th3/+}$ mice

To evaluate the activity of the anti-matriptase-2 antibody in reducing disease activity in β-thalassemic mice, 8-week-old *Hbb*$^{th3/+}$ mice were treated with weekly IP injections of RLYB331 for 8 weeks and compared with *Hbb*$^{th3/+}$ treated with a nonrelevant antibody of the same isotype (control IgG1). Control *Hbb*$^{th3/+}$ mice had elevated liver iron content compared to WT mice at the same age, but similar, rather than lower, hepcidin levels (Figure 1), indicating that, as previously reported,[25,26] hepcidin can still respond partially to iron status in this model of β-thalassemia intermedia. *Hbb*$^{th3/+}$ mice that received RLYB331 had increased liver *Hamp* messenger RNA (mRNA) (x2.24 on average; 95% confidence interval: 1.51-3.33) and increased serum hepcidin levels compared to controls (Figure 1A-B). RLYB331 was also effective in lowering serum iron levels (Figure 1C) and bringing liver iron content to values in the normal range (Figure 1D). Iron distribution in liver tissue sections from *Hbb*$^{th3/+}$ and WT mice was assessed following Perl's Prussian blue staining. Liver sections from *Hbb*$^{th3/+}$ mice treated with the control IgG1 displayed dense blue punctate staining, representing abnormally high levels of insoluble iron stores termed hemosiderin. RLYB331 notably decreased liver iron staining when compared with control IgG1 (Figure 1E). These results provide evidence that, by neutralizing matriptase-2 activity, RLYB331 represents a potent in vivo modulator of *Hamp* expression, serum iron availability and liver iron accumulation in the mouse model of thalassemia intermedia. Similar effects were observed when RLYB331 was given in combination with IP injections of RAP-536L, a modified activin receptor type IIB ligand trap, twice weekly, whereas, as expected from the different mode of action, treatment with RAP-536L alone did not affect hepcidin mRNA (Figure 1A), serum iron (Figure 1C), or liver iron content (Figure 1D-E). In the same line, despite increased erythropoietin level, combination of RLYB331 and darbepoetin alpha treatments resulted anyway in increased hepcidin mRNA expression and reduced liver iron content (supplemental Figure 2A-B).

RLYB331 ameliorates ineffective erythropoiesis in Hbb$^{th3/+}$ mice

To assess whether RLYB331 ameliorated ineffective erythropoiesis in *Hbb*$^{th3/+}$ mice, we identified the different stages of erythroid differentiation in the spleen and bone marrow by fluorescence-activated cell sorting analysis, using Ter119 and CD44 cell surface markers. The additional parameter, forward scatter, was used to discriminate between 5 distinct erythroid populations: (I) proerythroblasts, (II) basophilic, (III) polychromatic, (IV) orthochromatic cells and reticulocytes, and (V) RBCs. In the spleen, β-thalassemic erythroid cells exhibited increased cell proliferation (fractions II-IV) and reduced cell differentiation (fraction V) compared with those from normal mice (Figure 2A). However, when the fluorescence-activated cell sorting profiles of control *Hbb*$^{th3/+}$ mice and *Hbb*$^{th3/+}$ mice that received RLYB331 were compared, we observed that the latter group exhibited an improved profile, as indicated by a reduction in the number of cells in fractions III and IV, and an increase in terminally differentiated cells in fraction V (Figure 2A). RAP-536L was slightly less effective, with more moderate reduction of cells in fraction IV and increase of RBCs in fraction V. When administered together, RLYB331 and RAP-536L potentiated each other's effects, with more significant reduction of cells in fractions III and IV and increase in cells in fraction V (Figure 2A). In the bone marrow, β-thalassemic erythroid cells exhibited increased cell numbers in fractions II and III and reduced terminally differentiated cells in fraction V (Figure 2B). Both RLYB331 and RAP-536L increased cells in fraction V. Most notably, the 2 treatments combined potentiated each other's effects, with a distribution of erythroid precursors in the bone marrow of *Hbb*$^{th3/+}$ treated animals close to that seen in WT mice (Figure 2B). Collectively, these results demonstrate that the neutralizing matriptase-2 antibody RLYB331 significantly enhances in vivo maturation and differentiation of erythroid cells in the spleen and, to a lesser extent, in the bone marrow of *Hbb*$^{th3/+}$ mice. Interestingly, the combination of RLYB331 with RAP-536L entirely reverses ineffective erythropoiesis in the bone marrow.

RLYB331 limits the formation of toxic α-chain/heme aggregates and reduces ROS formation in Hbb$^{th3/+}$ mice

In β-thalassemia, the unpaired α-globin chains precipitate with heme, forming large, insoluble aggregates in RBCs, which increases ROS production and results in hemolysis. To test whether our treatments could reduce α-globin precipitates and thus improve the quality and survival of erythroid cells, we prepared membrane fractions from circulating RBCs. The amount of membrane-bound globins in *Hbb*$^{th3/+}$ control mice and *Hbb*$^{th3/+}$ mice that received the different treatments was assessed by triton acetic urea gel electrophoresis (Figure 3A; supplemental Figure 3). As expected, due to the unstable nature of free α-globins combined with relative β-globin deficiency, α-globin precipitates were present in large amounts in β-thalassemic erythrocytes. Compared with *Hbb*$^{th3/+}$ control mice, those treated with RAP-536L exhibited a moderate reduction in membrane-associated α-globin aggregates. Interestingly, α-globin was undetectable in *Hbb*$^{th3/+}$ mice treated with RLYB331 alone or in combination with RAP-536L, as well as in WT membrane fractions. β-globin was undetectable in membrane fractions from all groups as expected. These results indicate that RLYB331 neutralizes toxic free α-globin precipitates even more effectively than RAP-536L. To determine whether the lower amount of α globin precipitates reduced ROS formation, we added a ROS indicator, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acyl ester (CM-H_2DCFDA) to spleen and bone marrow cells. This compound permeates into the cells and is oxidized in the presence of ROS and free heme. Oxidation was detected by monitoring the increase in fluorescence by flow cytometry. The analysis of the Ter119+ erythroid populations in the spleen and the bone marrow indicated that ROS were increased in *Hbb*$^{th3/+}$ mice compared with controls. Remarkably, mice treated with RLYB331 significantly reduced ROS to near normal levels in the spleen (Figure 3B) but not in the bone marrow, in contrast to

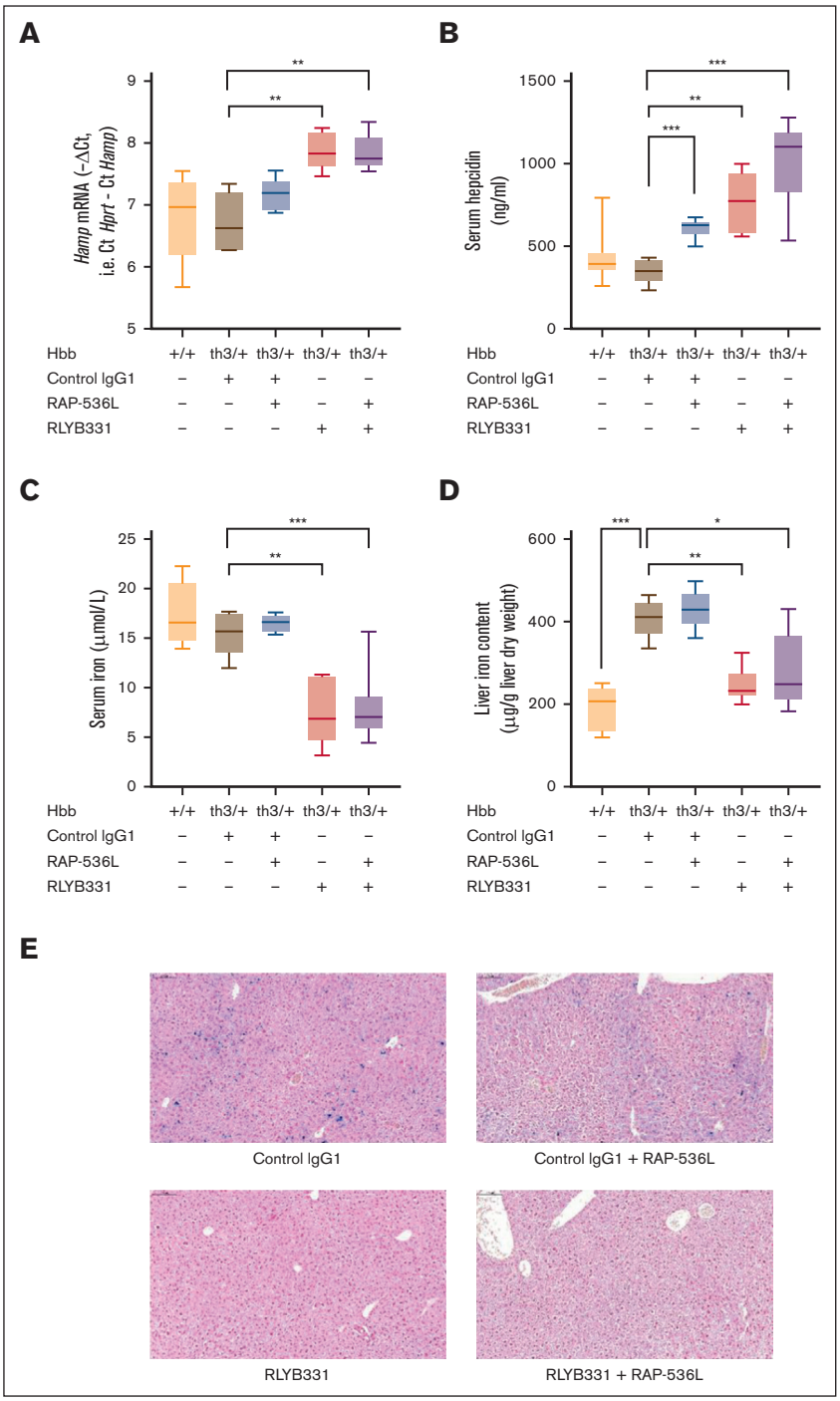

Figure 1. RLYB331 induces hepcidin expression and reduces iron overload in β-thalassemia intermedia (*Hbb*$^{th3/+}$) mice. Liver hepcidin (Hamp) mRNA expression levels (A), serum hepcidin levels (B), serum iron levels (C), and liver iron content (D) following treatment. (E) Representative images of Perl's Prussian blue-stained liver sections. *Hbb*$^{th3/+}$ (8-week-old males) mice were injected every week with 10 mg/kg of RLYB331 for 8 weeks. Controls received 10 mg/kg of a nonrelevant antibody of the same isotype (control IgG1). *Hbb*$^{th3/+}$ mice treated with RAP-536L received 10 mg/kg of ActRIIB-mFc twice weekly for 8 weeks. Values in WT mice are shown for comparison. Results are presented by Box plots with 6 to 10 mice per group. Statistical significance was determined by Brown-Forsythe and Welch analysis of variance (ANOVA) tests followed by Tamhane's T2 multiple comparisons tests and is indicated by the brackets (*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$).

mice treated with RAP-536L (Figure 3C). Further analysis using annexin V identified a large proportion of cells undergoing apoptosis in control *Hbb*$^{th3/+}$ mice compared with WT mice, both in the spleen and the bone marrow. RLYB331 significantly reduced the percentage of apoptotic (annexin V^{+}) erythroid cells in the spleen (Figure 3D) but not in the bone marrow (Figure 3E), in contrast to RAP-536L that significantly reduced apoptosis in the bone marrow but not in the spleen. Collectively, these results demonstrate that the neutralizing matriptase-2 antibody RLYB331 reduces ROS formation and apoptosis in the spleen of *Hbb*$^{th3/+}$ mice. However, association with RAP-536L is needed to reach similar effects in the bone marrow.

RLYB331 improves reticulocytosis and splenomegaly in Hbb$^{th3/+}$ mice

RLYB331 corrected reticulocytosis in *Hbb*$^{th3/+}$ mice even more effectively than RAP-536L alone (Figure 4A). Improvement of

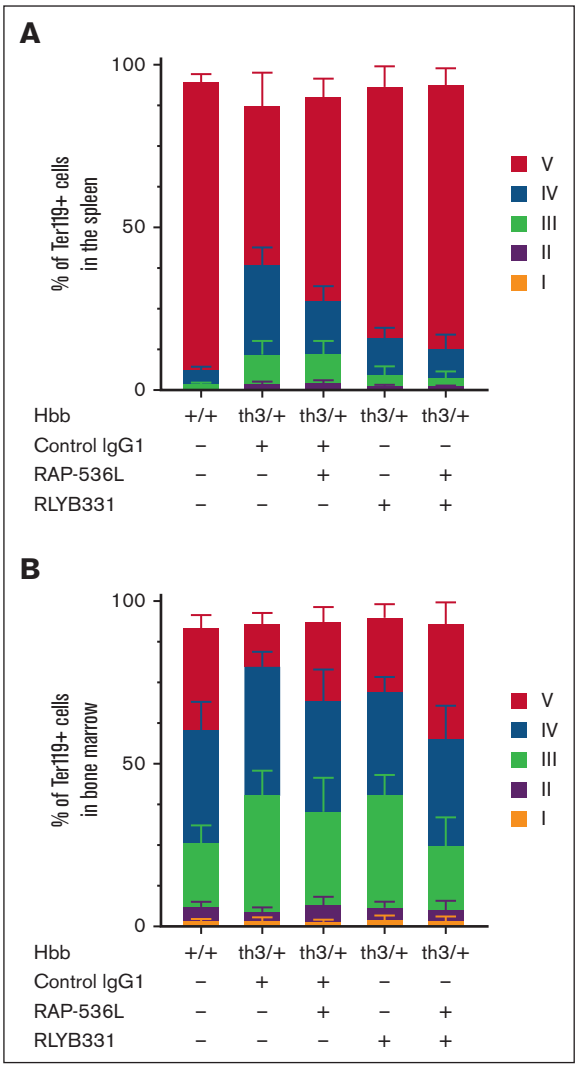

Figure 2. RLYB331 ameliorates ineffective erythropoiesis in β-thalassemia intermedia (*Hbb*$^{th3/+}$) mice. Flow cytometry analysis of the proportion of Ter119+ erythroid subpopulations in the spleen (A) and the bone marrow (B), using Ter119- and CD44-labeled antibodies. The additional parameter, forward scatter, was used to discriminate between 5 distinct stages of erythroid differentiation: (I) proerythroblasts, (II) basophilic, (III) polychromatic, (IV) orthochromatic cells and reticulocytes, and (V) RBCs. Analysis was performed in control *Hbb*$^{th3/+}$ mice and in *Hbb*$^{th3/+}$ mice that received the different treatments (RLYB331, RAP-536L, or the 2 combined). Data are presented by stacked bar graphs with mean ± standard deviation (SD) of 6 to 10 mice per group.

reticulocytosis was associated with a significant reduction in splenomegaly (Figure 4B). Morphological inspection of the spleen indicated that decreased splenomegaly was associated with improved organ architecture, showing a normalization of the relative proportion of the white and red pulp (data not shown). However, in contrast to RAP-536L, RLYB331 did not significantly increase hemoglobin levels, hematocrit, or RBC counts when compared to *Hbb*$^{th3/+}$ controls (Figure 4C-E). This is consistent with the fact that RLYB331 was less efficient in reducing apoptosis in the bone marrow. Notably, anemia end points were significantly improved when RLYB331 was given to *Hbb*$^{th3/+}$ mice in association with RAP-536L (Figure 4C-E) and in some extend with darbepoetin alpha (supplemental Figure 2C-E).

Discussion

Iron plays a major role in the deterioration of β-thalassemia. Indeed, the high levels of transferrin saturation and iron delivered to erythroid progenitors are associated with production of hemichromes that negatively affect erythropoiesis.[27-29] As patient's age, the size of the spleen increases and sequesters a growing number of damaged RBCs. Consequently, the number of erythroid progenitors expands in an attempt to compensate for the anemia, which negatively affects hepcidin expression in response to erythroferrone production[1] and causes increased intestinal iron absorption and recycling despite replete iron stores. Previous observations have shown that increasing hepcidin expression not only prevents or reduces iron overload but can also break this vicious cycle.[30] Given that matriptase-2 is the most important inhibitor of hepcidin gene expression, it was not surprising that suppression of *TMPRSS6* expression using antisense oligonucleotides[8,12,31] or small interfering RNA[9,14,32] in β-thalassemic mice increased hepcidin and decreased iron loading. Interestingly, these treatments also improved erythropoiesis and anemia. Based on these findings, it has been suggested that the use of these treatments could benefit thalassemic patients and there are several ongoing clinical trials to assess their efficacy and safety.[15]

In this study, we considered an alternative approach to reach similar effects. This approach consists in blocking the protease activity of matriptase-2 via a monoclonal antibody binding to the serine protease active site. Antibodies constitute the most successful and safest class of biological pharmaceuticals today.[33] They are often preferred over small molecules because they are natural products, combining high binding affinities and high target specificity with long in vivo half-lives, and generally have superior safety profiles. To obtain a fully human anti-matriptase 2 antibody, transgenic mice engineered to express the entire human immunoglobulin variable-gene repertoire[17] were used. These mice mount an effective immune response when challenged, producing high-affinity, fully-human antibody. The use of fully-human antibodies in patients avoids or reduces immunogenicity problems and thus is a favorable modality for long-term treatment of chronic diseases, which is particularly important for a disease such as β-thalassemia. RLYB331 was identified through a multistep screening cascade and blocks the protease activity of human and murine matriptase-2. When administered weekly to *Hbb*$^{th3/+}$ mice, it was shown to induce hepcidin expression, reduce iron loading, prevent the formation of toxic α-chain/heme aggregates, reduce ROS formation, and improve reticulocytosis and splenomegaly. However, the ineffective erythropoiesis associated with this model of β-thalassemia was not entirely corrected and, after 8 weeks of treatment, RBC counts as well as hemoglobin and hematocrit levels were still not improved.

We thus decided to increase the effectiveness of RLYB331 in β-thalassemia treatment through a combinatorial approach, as initiated recently by others.[12] We administered the matriptase-2 neutralizing antibody in combination with RAP-536L, a ligand-trapping fusion protein known to sequestrate ligands of the TGF-β superfamily before they interact with their receptor,[7] thus inhibiting the Smad2/3 signal transduction cascade which plays an

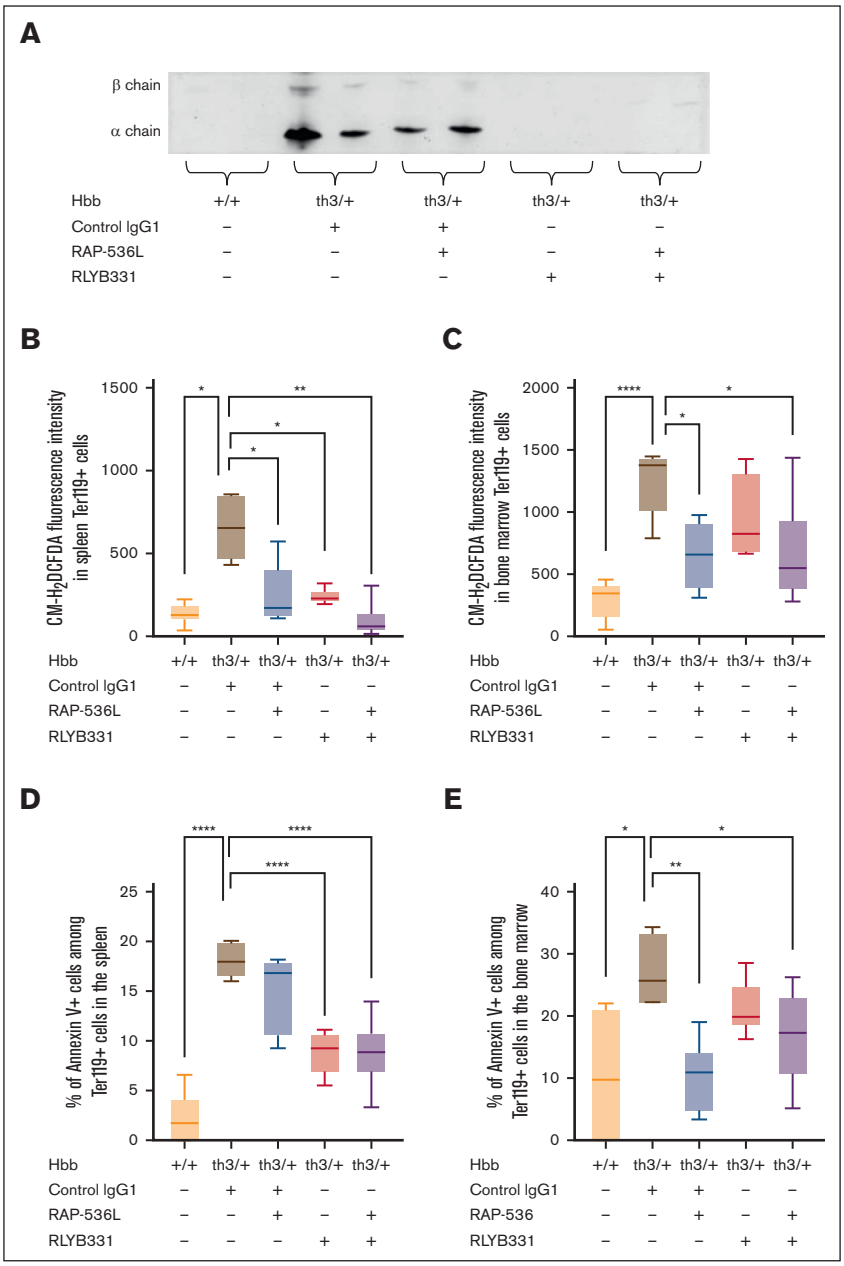

Figure 3. RLYB331 limits the formation of toxic α-chain/heme aggregates and reduces ROS formation in *Hbb*$^{th3/+}$ mice. (A) Triton/acetic acid/urea (TAU) gel electrophoresis of membrane-bound globins in RBCs from WT mice, control *Hbb*$^{th3/+}$ mice, and *Hbb*$^{th3/+}$ mice that received the different treatments (RLYB331, RAP-536L, or the 2 combined). Median fluorescence intensity of spleen (B) and bone marrow (C) Ter119^{+} erythroid cells generated following incubation with the ROS indicator CM-H_2DCFDA. Mean percentage of apoptotic (annexin V+) Ter119+ erythroid cells in the spleen (D) and bone marrow (E). Results are presented by Box plots with 6 to 10 mice per group. Statistical significance was determined by Brown-Forsythe and Welch ANOVA tests followed by Tamhane's T2 multiple comparisons tests and is indicated by the brackets (*$P \leq .05$; **$P \leq .01$; ****$P \leq .0001$).

inhibitory role in terminal erythroid differentiation. As expected from previous studies, RAP-536L treatment alone did not prevent iron overload but significantly reduced apoptosis in the erythroid populations of the bone marrow, normalized RBC counts, and improved hemoglobin and hematocrit levels. Interestingly, the association RLYB331 with RAP-536L entirely reversed the β-thalassemia phenotype in *Hbb*$^{th3/+}$ mice and corrected simultaneously iron overload, ineffective erythropoiesis, splenomegaly, and hematological parameters.

Our study provides compelling evidence to support the notion that RLYB331 holds important promise in significantly enhancing the treatment efficacy of luspatercept for individuals suffering from NTDT. The unique mechanisms of action of both RLYB331 and luspatercept complement each other in a manner that fosters superior patient outcomes. Luspatercept promotes erythropoiesis and RBC production, effectively mitigating anemia. In conjunction, RLYB331 exerts its therapeutic influence not only by correcting the iron overload but also by ameliorating the erythroid progenitor maturation in the spleen. The synergistic effects of combining these 2 therapeutic agents are poised to improve the management and care of patients afflicted with this medical condition.

Another interesting approach to enhance the effectiveness of RLYB331 in the treatment of β-thalassemia could involve combining it with erythropoiesis enhancing agents such as darbepoetin alpha. This combination aims to correct the iron overload but also to stimulate the production of RBCs and effectively address anemia. Building on the findings of our previous study,[34] it is worth noting that higher levels of erythropoietin did not prevent

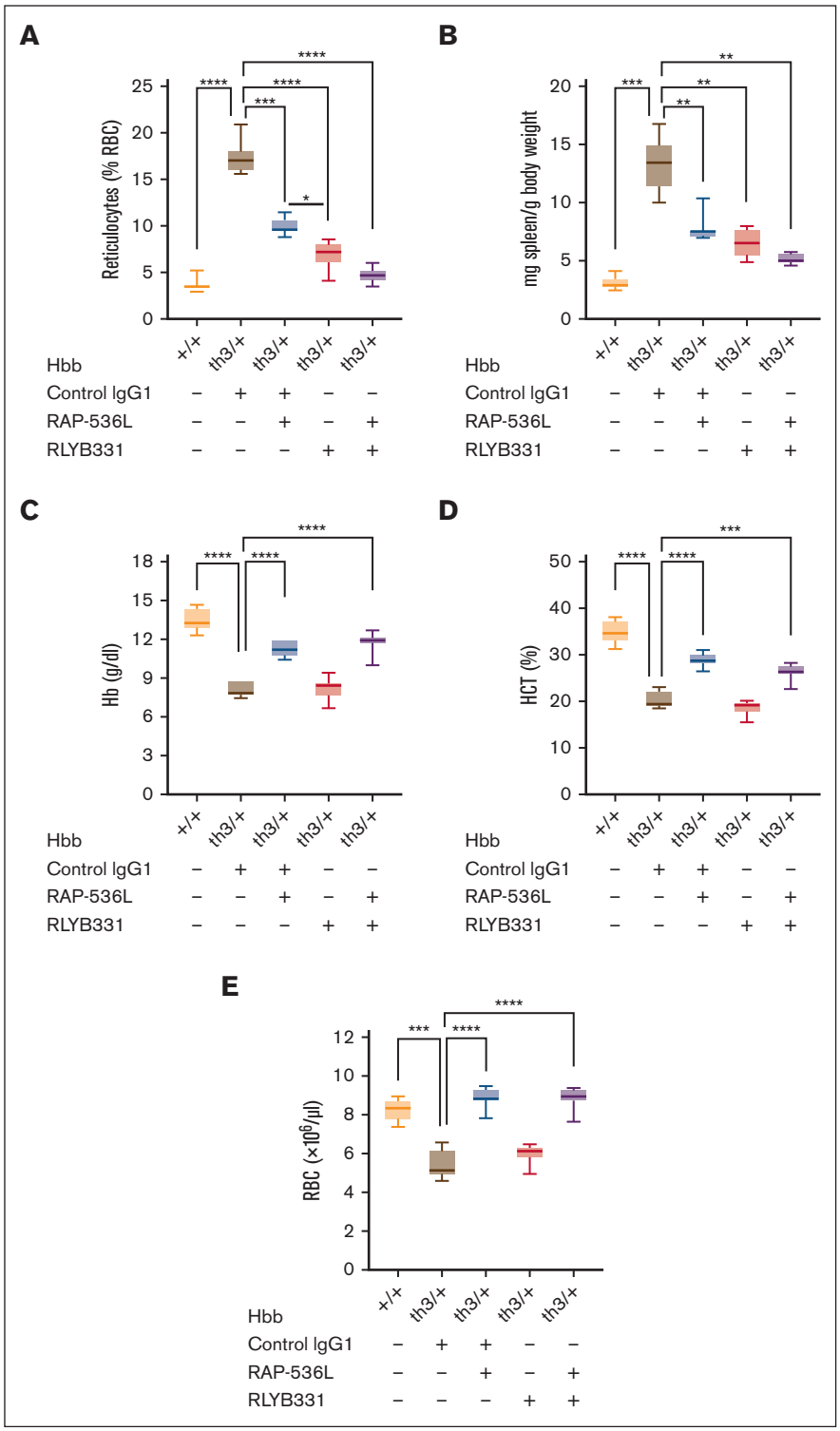

Figure 4. RLYB331 improves reticulocytosis and splenomegaly in *Hbb*$^{th3/+}$ mice. Reticulocyte count as a percentage of total RBCs (A), spleen index (B), hemoglobin levels (C), hematocrit (D), and RBCs (E) in control *Hbb*$^{th3/+}$ mice and *Hbb*$^{th3/+}$ mice that received the different treatments (RLYB331, RAP-536L, or the 2 combined). Results are presented by Box plots with 6 to 10 mice per group. Statistical significance was determined by Brown-Forsythe and Welch ANOVA tests followed by Tamhane's T2 multiple comparisons tests and is indicated by the brackets (**$P \leq .01$; ***$P \leq .001$; ****$P \leq .0001$).

the elevation of hepcidin levels induced by matriptase-2 inhibition and therefore still corrected the iron overload.

It is important to acknowledge that this combined approach also demonstrates some improvement in addressing anemia. However, further studies are warranted to determine whether an optimal dosage can be identified to further enhance its effectiveness in managing anemia.

Altogether, our study confirms that combinatorial treatments with agents eliciting different but complementary mechanisms of action are superior to single treatments.

RLYB331 is a first-in-class fully human monoclonal antibody that specifically neutralizes matriptase-2. The very specific inhibitory role of matriptase-2 on hepcidin production makes it, as shown in this study, a promising option for the management of secondary iron overload in β-thalassemia, while minimizing the risk of any significant off-target effect and safety concerns. By restoring iron homeostasis in *Hbb*$^{th3/+}$ mice, this antibody markedly reduced levels of membrane-associated α-globin aggregates in erythrocytes, the main cause of erythrocytic damage and hemolysis in β-thalassemia. This antibody could therefore also be helpful for the treatment of primary iron overload in diseases with insufficient hepcidin synthesis such as genetic hemochromatosis. Being fully human, it can be rapidly reformatted into therapeutics that can be assessed in clinical trials for these important diseases. The association of RLYB331 with RAP-536L ameliorates not only iron overload but also anemia. Therefore, after assessing the effectiveness of RLYB331 treatment alone and in combination with luspatercept in patients, it might be considered to develop a multifunctional molecule consisting of the fusion of the RLYB331 IgG backbone structure with the ligand-trapping protein moiety containing the extracellular domain of activin receptor type IIB, similar to luspatercept.[18]

This would allow administration of a single medication addressing simultaneously the different pathophysiological aspects of the disease, which may provide a significant health benefit to patients with β-thalassemia.

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Authorship

Contribution: M.W., A. Palin, and A.B., performed experiments, analyzed and discussed results, and edited the manuscript; M.L., M. Berger, J. Papworth, L.B., B.G., N.R., J. Paterson, A. Poindron, E.S., E.C., R.H., M.C., V.P., M. Bichon, C.B.-F., C.L., and A.L. performed experiments, and analyzed and discussed results; O.G. and A.F. performed experiments; V.G. and I.T. designed research plans, and analyzed and discussed data; H.C. and M.-P.R. discussed data and wrote the manuscript; D.M. designed research, performed experiments, analyzed and discussed data, and wrote the manuscript; and all authors reviewed and approved the final manuscript.

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