

Featured Article

# Hematologic safety of chronic brain-penetrating erythropoietin dosing in APP/PS1 mice

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## Abstract

**Introduction:** Low blood-brain barrier (BBB) penetration and hematopoietic side effects limit the therapeutic development of erythropoietin (EPO) for Alzheimer's disease (AD). A fusion protein of EPO and a chimeric monoclonal antibody targeting the mouse transferrin receptor (cTfRMAB) has been engineered. The latter drives EPO into the brain via receptor-mediated transcytosis across the BBB and increases its peripheral clearance to reduce hematopoietic side effects of EPO. Our previous work shows the protective effects of this BBB-penetrating EPO in AD mice but hematologic effects have not been studied. Herein, we investigate the hematologic safety and therapeutic effects of chronic cTfRMAB-EPO dosing, in comparison to recombinant human EPO (rhu-EPO), in AD mice.

**Methods:** Male APP<sup>sw</sup>e PSEN1<sup>de9</sup> (APP/PS1) mice (9.5 months) were treated with saline (n = 11), and equimolar doses of cTfRMAB-EPO (3 mg/kg, n = 7), or rhu-EPO (0.6 mg/kg, n = 9) 2 days/week subcutaneously for 6 weeks, compared to saline-treated wild-type mice (n = 10). At 6 weeks, exploration and memory were assessed, and mice were sacrificed at 8 weeks. Spleens were weighed, and brains were evaluated for amyloid beta (A $\beta$ ) load and synaptophysin. Blood was collected at 4, 6 and 8 weeks for a complete blood count and white blood cells differential.

**Results:** cTfRMAB-EPO transiently increased reticulocyte counts after 4 weeks, followed by normalization of reticulocytes at 6 and 8 weeks. rhu-EPO transiently increased red blood cell count, hemoglobin and hematocrit, and significantly decreased mean corpuscular volume and reticulocytes at 4 weeks, which remained low at 6 weeks. At 8 weeks, a significant decline in red blood cell indices was observed with rhu-EPO treatment. Exploration and cognitive deficits were significantly worse in APP/PS1-rhu-EPO mice. Both cTfRMAB-EPO and rhu-EPO decreased 6E10-positive brain A $\beta$  load; however, cTfRMAB-EPO and not rhu-EPO selectively reduced brain A $\beta$ <sub>1-42</sub> and elevated synaptophysin expression.

**Discussion:** Chronic treatment with cTfRMAB-EPO results in better hematologic safety, behavioral, and therapeutic indices compared with rhu-EPO, supporting the development of this BBB-penetrable EPO analog for AD.

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## Keywords:

Blood-brain barrier; Safety; Hematology; Erythropoietin; Alzheimer's disease; Transferrin receptor; Monoclonal antibody

## 1. Introduction

The most widely studied hypothesis to elucidate Alzheimer's disease (AD) pathogenesis is the amyloid hypothesis [1], and most drug candidates under clinical development for AD are anti-amyloid beta (A $\beta$ ) agents [2]. However, anti-A $\beta$  clinical trials have been unsuccessful with respect to slowing progression of dementia [3]. Given the strong evidence supporting the role of A $\beta$  accumulation in the AD brain [1], failure of anti-A $\beta$  agents does not necessarily disprove the amyloid hypothesis but perhaps suggests that A $\beta$  load reduction alone may not be enough to reverse the neurite dystrophy and dementia of AD [4]. Because A $\beta$  plaque deposition occurs decades before clinical AD [5], therapeutic agents that enhance the repair of dystrophic neurites after plaque reduction may aid in the reversal of dementia. Therefore, a therapy that combines A $\beta$  load reduction with reversal of neuronal damage may yield cognitive improvement in AD.

Erythropoietin (EPO), a hematopoietic growth factor, is a unique therapeutic candidate for AD because it reduces AD pathology and produces neurotrophic effects to repair neuronal damage in AD [6]. However, limited blood-brain barrier penetration and hematopoietic side effects associated with chronic EPO treatment limit its clinical applicability as a neurotherapeutic [7–10]. To overcome these limitations, an IgG-EPO fusion protein was engineered, where the IgG domain is a rat/mouse chimeric monoclonal antibody against the mouse TfR (cTfRMAB). Fusion of EPO to cTfRMAB offers dual functions: (1) it enables binding to the blood-brain barrier TfR for EPO brain delivery and (2) it enables peripheral TfR binding of the fusion protein resulting in faster clearance of IgG-EPO from blood as compared with EPO, which reduces the hematopoietic effects of EPO [6,7,11]. This approach thus drives EPO into the brain in parallel with a reduction in peripheral side effects of EPO.

In experimental Parkinson's disease, a 1 mg/kg dose of cTfRMAB-EPO administered 3 days/week for 3 weeks via the intravascular (IV) route resulted in a 12% increase in hematocrit [12]. For long-term treatment, the extravascular (EV) route, such as subcutaneous administration, is preferred over the IV route due to the ease of administration. However, lower bioavailability after EV dosing necessitates higher EV injection doses to achieve therapeutic brain concentrations comparable with IV dosing, and the typical EV dose of IgG-based fusion proteins is higher and ranges between 3 and 5 mg/kg [13–15]. We recently showed that chronic treatment with a 3 mg/kg EV dose of cTfRMAB-EPO reduces A $\beta$  load, neuroinflammation, synaptic loss, and spatial memory deficits in the APPswe PSEN1dE9 (APP/PS1) mice [13]. However, the effect of chronic EV doses of cTfRMAB-EPO on hematologic indices is unknown. The aim of the present study was therefore to investigate the hematologic safety and therapeutic effects of the brain-penetrating EPO, in comparison with treatment with

recombinant human EPO (rhu-EPO), after subcutaneous 6-week dosing in APP/PS1 mice. At 8 weeks, behavior, brain A $\beta$  load, and neuronal function were assessed. Hematologic indices were measured at 4, 6, and 8 weeks after treatment initiation.

## 2. Methods

### 2.1. Fusion protein

cTfRMAB-EPO was produced in stably transfected Chinese hamster ovary cells cultured in serum-free medium and purified from conditioned medium by protein-G affinity chromatography [11]. Both rhu-EPO (Creative BioMart) and cTfRMAB-EPO were formulated in 98 mM glycine, 148 mM NaCl, 28 mM Tris, 0.01% polysorbate 80, pH = 5.5, and stored at  $-80^{\circ}\text{C}$ .

### 2.2. Mouse treatment

All animal procedures followed the "Principles of Laboratory Animal Care" (NIH Publication No. 85-23), were approved by the University of California, Irvine, Institutional Animal Care and Use Committee, and followed the ARRIVE Guidelines for animal experiment reporting. Mice had constant access to food and water and were maintained on a 12-hour light/12-hour dark cycle. Male heterozygous APP/PS1 mice (9.5 months, strain B6C3-Tg APPswe, PSEN1dE9, 85Dbo/Mmjax, Jackson Laboratories) were injected subcutaneously twice per week, for 6 weeks (Fig. 1A) with saline (n = 11), cTfRMAB-EPO (3 mg/kg, n = 7), or rhu-EPO (0.6 mg/kg which is equivalent to 60,000 IU/kg since 1IU rhu-EPO  $\sim$  10 ng, n = 9). These are equimolar doses of cTfRMAB-EPO and rhu-EPO because the EPO domain constitutes 20% of cTfRMAB-EPO [11]. Age-matched wild-type (WT) C57BL/6J littermate mice (n = 10) were treated similarly with equivalent volume of saline. Mice were weighed weekly and monitored for immune responses (prostrate, unresponsive, or scruffy appearance) after injection. Eight weeks after treatment initiation, mice were anesthetized with Euthasol (50 mg/kg, i.p.), transcardially perfused with phosphate-buffered saline (PBS), and the brain and spleen were harvested.

### 2.3. Complete blood count analysis

Blood was collected via the retro-orbital sinus at 4, 6, and 8 weeks for complete blood count including white blood cell differential (Molecular Diagnostic Services, Inc.).

### 2.4. Behavioral testing

Behavior analysis was performed before and after 6 weeks of treatment. Locomotion and exploration were assessed by the open-field test as described previously [13]. Movements of animals, after placing in a white open box

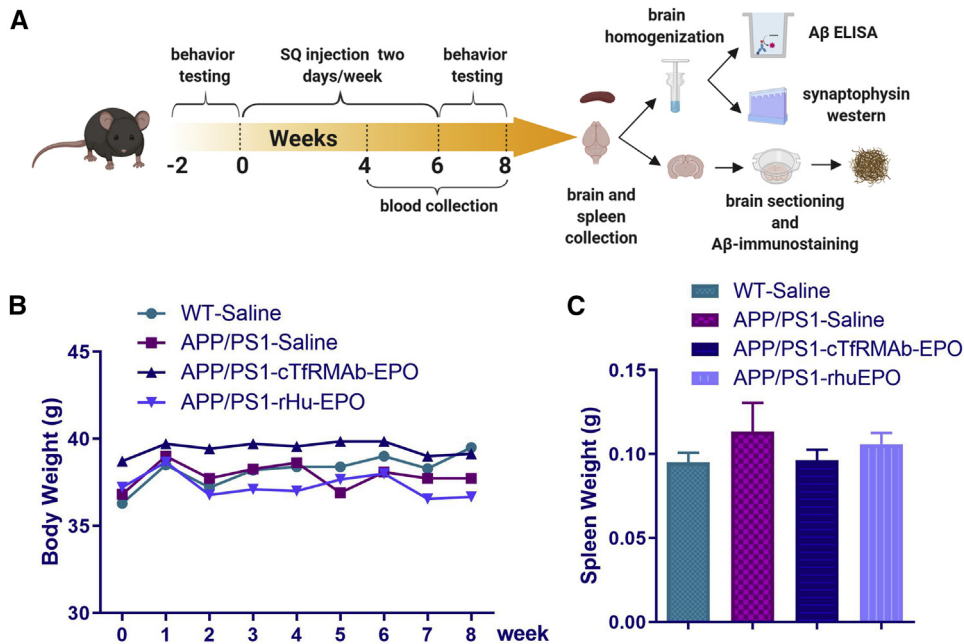


Fig. 1. Body and spleen weight changes after chronic treatment with cTfRMAb-EPO and rhu-EPO. Schematic of the injection and blood collection timeline (A). Panel A was created with BioRender.com. Mice were weighed weekly up to 8 weeks after treatment initiation. No significant difference was observed in the weight of the mice between the experimental groups (B). No significant difference in spleen weight was observed between the experimental groups (C). Data are presented as mean  $\pm$  SEM of 7-11 mice per group. One-way ANOVA with Holm-Sidak's post hoc test was used to compare with WT-saline controls. Abbreviations: EPO, erythropoietin; WT, wild-type.

(72 cm  $\times$  72 cm with 36 cm walls), were recorded for 5 min to measure (a) mean speed, (b) resting time, and (c) total distance [13]. Time in the center was also recorded as an indicator of anxiety-related behavior [16].

Spatial memory was assessed using the modified Y-maze as previously described [13]. Briefly, during the training phase, one arm of the Y-maze was blocked and the animal was placed in the start arm and was allowed to explore two open arms for 8 min. After 30 min, during the testing phase, the animal was placed back into the start arm with access to all the three arms for 8 min. Percentage entries into novel arm was calculated as a measure of spatial memory. All analysis was performed using the SMART Video Tracking Software (Panlab, Harvard Apparatus).

Recognition memory was assessed using the novel object recognition (NOR) test as previously described [14]. After a two-day habituation in a white open box for 5 min, mice were exposed to two identical objects placed equidistant at two opposite positions in the box for 10 min on day 3. One hour later, recognition memory was tested by exposing the mouse to one familiar and one new object for 10 min. Recognition index was calculated as follows: [time exploring new object/(time exploring new object + time exploring familiar object)]  $\times$  100%.

## 2.5. Cryosectioning

Right cerebral hemispheres were immersion-fixed in 4% paraformaldehyde followed by cryoprotection in sequential

10%-, 20%-, and 30%-sucrose solution at 4°C, frozen, mounted in Tissue-Tek OCT (Fisher Scientific) and sectioned into 20  $\mu$ m sagittal sections with a cryostat (Micron Instruments). Three sagittal sections (600  $\mu$ m apart) incorporating both the cortex and the hippocampus were stained per mouse.

## 2.6. Amyloid beta immunofluorescence

Sections were washed in PBS, incubated in 70% formic acid for 10 min at room temperature (RT), and blocked with 0.5% bovine serum albumin in PBS containing 0.3% TritonX-100 for 1 h at RT followed by overnight incubation with 1  $\mu$ g/mL of Alexa Fluor 488-conjugated 6E10 MAb (BioLegend) with 0.3% TritonX-100 in PBS at 4°C [13]. Sections were cover-slipped with VectaMount aqueous mounting media (Vector Laboratories) and imaged using the Leica TCS SP5 Confocal Microscope. The entire hippocampus and two regions in the cortex of each section were imaged at 10X magnification. Images were analyzed using NIH ImageJ (version 1.52a) for the number of positive stains/mm<sup>2</sup> of the brain and stain-positive area which was expressed as a percentage of the total analyzed area. All images were analyzed by two observers blinded to the experimental groups.

## 2.7. A $\beta$ (1-42) ELISA

Frozen left cerebral hemispheres without the cerebellum were pulverized on dry ice and homogenized in 10 volumes

of Tris-buffered saline (50 mM Tris-Cl, pH 7.6; 150 mM NaCl, 5 mM EDTA, 2 mM 1,10-phenanthroline) with Roche complete EDTA-free Mini protease inhibitor by a mechanical homogenizer (Waverly). The homogenate was centrifuged at 100,000g for 1 h at 4°C. The pellet was suspended in 10 volumes of homogenization buffer (5 M guanidine HCl, 0.05 M Tris, pH 8) and agitated for 2.5 h at RT. After centrifugation at 20,800 g for 15 min, a 50  $\mu$ L aliquot of the supernatant was diluted 1:50 using a dilution buffer (0.02 M Tris, 0.15 M NaCl, 1 mM EDTA, 1% TritonX-100, pH 7.5) and protein concentration was measured using the bicinchoninic acid kit (Pierce Chemical Co.). Brain homogenate was further diluted 1:500 for A $\beta$ (1–42) detection using the Legend Max<sup>TM</sup>  $\beta$ -Amyloid x-42 ELISA Kit (BioLegend). Absorbance (OD) was measured at 620 nm. Standard curves were fit to the 4-parameter logistic regression curve and A $\beta$ (1–42) levels were calculated and normalized based on the protein amount in the brain samples.

## 2.8. Western blotting

Pulverized brain samples were lysed in a radioimmuno-precipitation assay buffer with Pierce Protease Inhibitor (ThermoFisher Scientific). Blots were probed with an anti-synaptophysin mouse MAb (1:1000 dilution; Santa Cruz Biotechnology) at 4°C overnight. Membranes were exposed to mouse IgG kappa binding protein conjugated to Horse-radish Peroxidase (Santa Cruz Biotechnology), followed by chemiluminescence detection (Fisher Scientific). Anti- $\beta$ -actin antibody (1:1000 dilution; Santa Cruz Biotechnology) was used as loading control. Chemiluminescence was detected using the Azure C500 gel imager (Azure Biosystems), and ImageJ was used for quantification of Western blot signals.

## 2.9. Statistical analysis

Data are represented as mean  $\pm$  SEM and all statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc.). Normality was determined using the Kolmogorov-Smirnov test. For normal data, one-way ANOVA with Holm-Sidak's post hoc test (for equal variance) or Welch's ANOVA with Dunnett's T3 multiple comparisons test (for unequal variance) were used. Kruskal-Wallis with Dunn's multiple comparisons test was used for non-normal data. Body weights were analyzed using two-way repeated-measures ANOVA with Holm-Sidak's multiple comparisons test. For NOR and Y-maze, a composite z-score was used to determine treatment effect on overall memory deficits [17]. For this, individual mouse values (recognition index and % entries in novel arm) were normalized to a z-score using the mean and standard deviation of the WT group. The composite memory z-score was the average of the z-scores of each test (NOR and Y-maze) and the z-score value was compared to a

Table 1

Red blood cell indices and platelet count of mice treated with saline, cTRMAB-EPO, or rhu-EPO for 6 weeks followed by 2-week recovery period

Parameter	4 weeks			6 weeks			8 weeks			
	WT	APP/PS1	APP/PS1	WT	APP/PS1	APP/PS1	WT	APP/PS1	APP/PS1	
	saline	saline	cTRMAB-EPO	saline	saline	cTRMAB-EPO	saline	saline	cTRMAB-EPO	
RBC ( $10^3/\mu$ L)	8.9 $\pm$ 0.1	9.7 $\pm$ 0.2*	9.8 $\pm$ 0.2*	12.3 $\pm$ 0.2*	8.9 $\pm$ 0.4	9.3 $\pm$ 0.5	8.5 $\pm$ 0.2	9.5 $\pm$ 0.2	8.2 $\pm$ 0.6	4.6 $\pm$ 0.7 <sup>†</sup>
HGB (g/dL)	13.6 $\pm$ 0.2	14.3 $\pm$ 0.5	14.4 $\pm$ 0.3	18.3 $\pm$ 0.5 <sup>†</sup>	13.8 $\pm$ 0.4	14.2 $\pm$ 0.6	12.9 $\pm$ 0.3	14.1 $\pm$ 0.3	12.1 $\pm$ 0.9	6.8 $\pm$ 1.1 <sup>†</sup>
HCT (%)	40.0 $\pm$ 0.8	41.5 $\pm$ 1.3	43.0 $\pm$ 1.6	49.9 $\pm$ 1.8 <sup>†</sup>	38.4 $\pm$ 1.2	39 $\pm$ 1.7	37.1 $\pm$ 1.1	40.0 $\pm$ 0.5	35.4 $\pm$ 2.4	18.5 $\pm$ 2.9 <sup>†</sup>
Platelet ( $10^3/\mu$ L)	883 $\pm$ 17	860 $\pm$ 111	892 $\pm$ 58	792 $\pm$ 64	1003 $\pm$ 32	917 $\pm$ 30	977 $\pm$ 43	908 $\pm$ 88	1003 $\pm$ 19	835 $\pm$ 173
MCV (fl)	45 $\pm$ 0.6	43 $\pm$ 0.6	44 $\pm$ 0.6	41 $\pm$ 1*	43 $\pm$ 0.56	42 $\pm$ 0.33	44 $\pm$ 0.6	42 $\pm$ 0.3	43 $\pm$ 0.6	39 $\pm$ 0.9 <sup>†</sup>
Total Retic (%)	3.8 $\pm$ 0.1	3.9 $\pm$ 0.1	7.9 $\pm$ 0.8 <sup>†</sup>	1.2 $\pm$ 0.2 <sup>†</sup>	4.6 $\pm$ 0.2	4.9 $\pm$ 1.4	3.6 $\pm$ 0.1	3.9 $\pm$ 0.1	2.8 $\pm$ 0.2	1.6 $\pm$ 0.4 <sup>†</sup>

Abbreviations: RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; Retic, reticulocytes; WT, wild-type.

Data are represented as mean  $\pm$  SEM.

\*  $p < .05$  compared to WT-saline.

<sup>†</sup>  $p < .01$  compared to WT-saline.

<sup>‡</sup>  $p < .001$  compared to WT-saline.

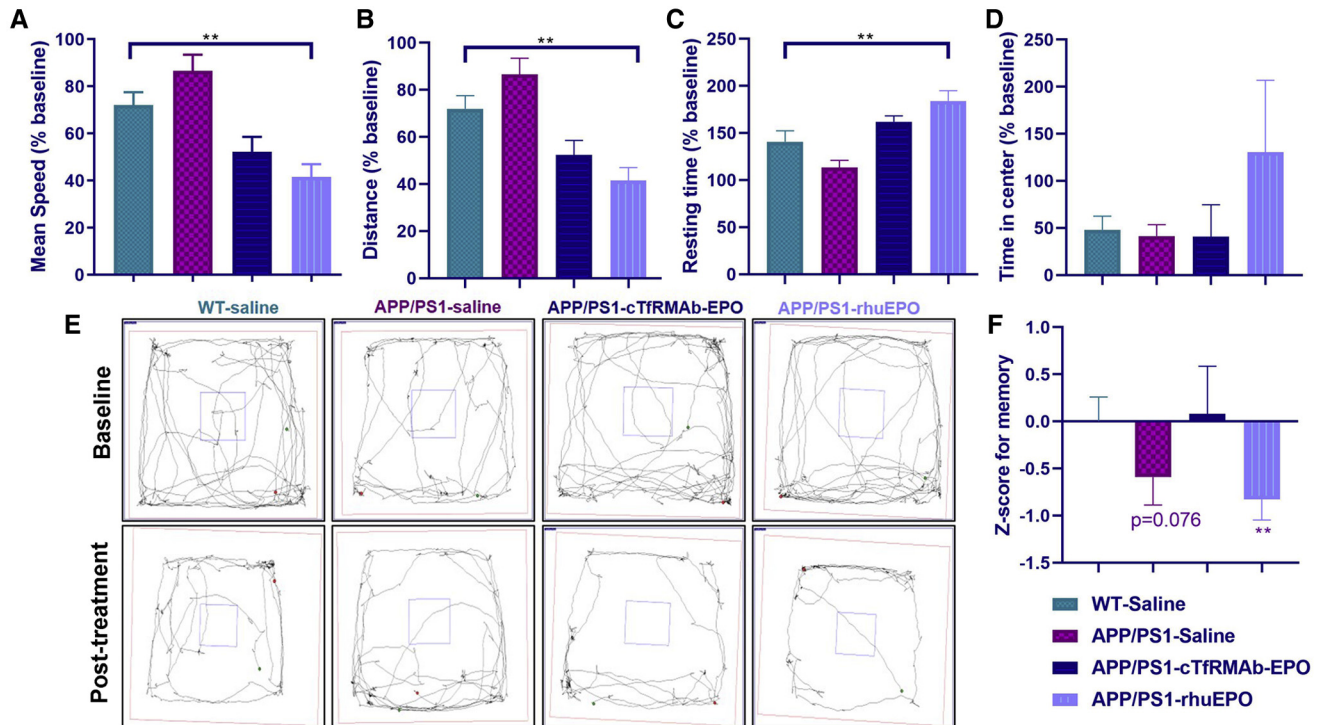


Fig. 2. Behavior analysis after chronic treatment with cTfRMAB-EPO and rhu-EPO. For the open-field (OF) test, the results at 6 weeks after treatment initiation were expressed as a percentage of baseline. rhu-EPO-treated APP/PS1 mice had significantly lower mean speed (A) and total distance (B) compared with WT-saline mice. Resting time in the APP/PS1-rhu-EPO mice was significantly higher than that in WT-saline mice (C). Time in the center was not significantly different between the experimental groups (D). Representative trajectories of saline-treated WT and saline-, cTfRMAB-EPO-, and rhu-EPO-treated APP/PS1 mice during the OF test (E). Composite memory z-scores for the recognition index during the NOR and % entries into novel arm during the Y-maze (F). Z-scores were significantly lower for APP/PS1-rhu-EPO mice and borderline significant for APP/PS1-saline mice. Data are presented as mean  $\pm$  SEM of 7-11 mice per group. One-way ANOVA with Holm-Sidak's post hoc test was used to compare to the WT-saline controls for OF test, and one-sample t-test with a hypothesized mean = 0 for the z-score. **\*\*P** < .01. Abbreviations: EPO, erythropoietin; NOR, novel object recognition; WT, wild-type.

hypothesized value = 0 (indicating no difference from the WT group). A two-tailed  $P < .05$  was considered statistically significant.

### 3. Results

#### 3.1. Body-weight and hematological profile

All mice survived and no signs of an immune response were observed. No significant difference was observed in the weight of the mice either at the beginning or end of the study between the experimental groups. The body weights of the mice at the beginning of the study were  $36.6 \pm 1.4$ ,  $36.8 \pm 1.7$ ,  $38.7 \pm 1.8$ , and  $37.2 \pm 1.4$  g in the WT-saline, APP/PS1-saline, APP/PS1-cTfRMAB-EPO, and APP/PS1-rhu-EPO mice, respectively (Fig. 1B). The body weights at 8 weeks were  $39.8 \pm 1.3$ ,  $37.7 \pm 1.7$ ,  $39.1 \pm 1.8$ , and  $36.7 \pm 1.6$  g in the WT-saline, APP/PS1-saline, APP/PS1-cTfRMAB-EPO, and APP/PS1-rhu-EPO mice, respectively (Fig. 1B). No significant difference was observed in the spleen weights, and the spleen weights were as follows:  $0.095 \pm 0.006$ ,  $0.11 \pm 0.017$ ,  $0.096 \pm 0.006$ , and  $0.11 \pm 0.007$  g in the WT-saline,

APP/PS1-saline, APP/PS1-cTfRMAB-EPO, and APP/PS1-rhu-EPO groups, respectively (Fig. 1C).

Four weeks after treatment initiation, red blood cell (RBC) counts in the APP/PS1-saline, APP/PS1-cTfRMAB-EPO, and APP/PS1-rhu-EPO mice were significantly higher by 9%, 10%, and 38%, respectively, compared with WT-saline mice (Table 1). In APP/PS1-cTfRMAB-EPO mice, reticulocytes increased by 108% compared with WT-saline mice. Most RBC indices were significantly altered by rhu-EPO treatment at 4 weeks. In APP/PS1-rhu-EPO mice, there was a significant reduction in the mean corpuscular volume (MCV) by 9% and total reticulocytes by 68%, along with a significant increase in hemoglobin and hematocrit by 35% and 25%, respectively, compared to WT-saline mice. The MCV and reticulocyte counts continued to be low at 6 weeks, and there was a 12% and 84% reduction in the MCV and total reticulocytes, respectively, in the APP/PS1-rhu-EPO mice compared with WT-saline mice (Table 1). Notably, the levels of RBC, hemoglobin, hematocrit, MCV, and reticulocytes in APP/PS1-rhu-EPO mice were significantly lower than those in WT-saline mice by 46%, 47%, 50%, 11%, and 56%, respectively, at 8 weeks

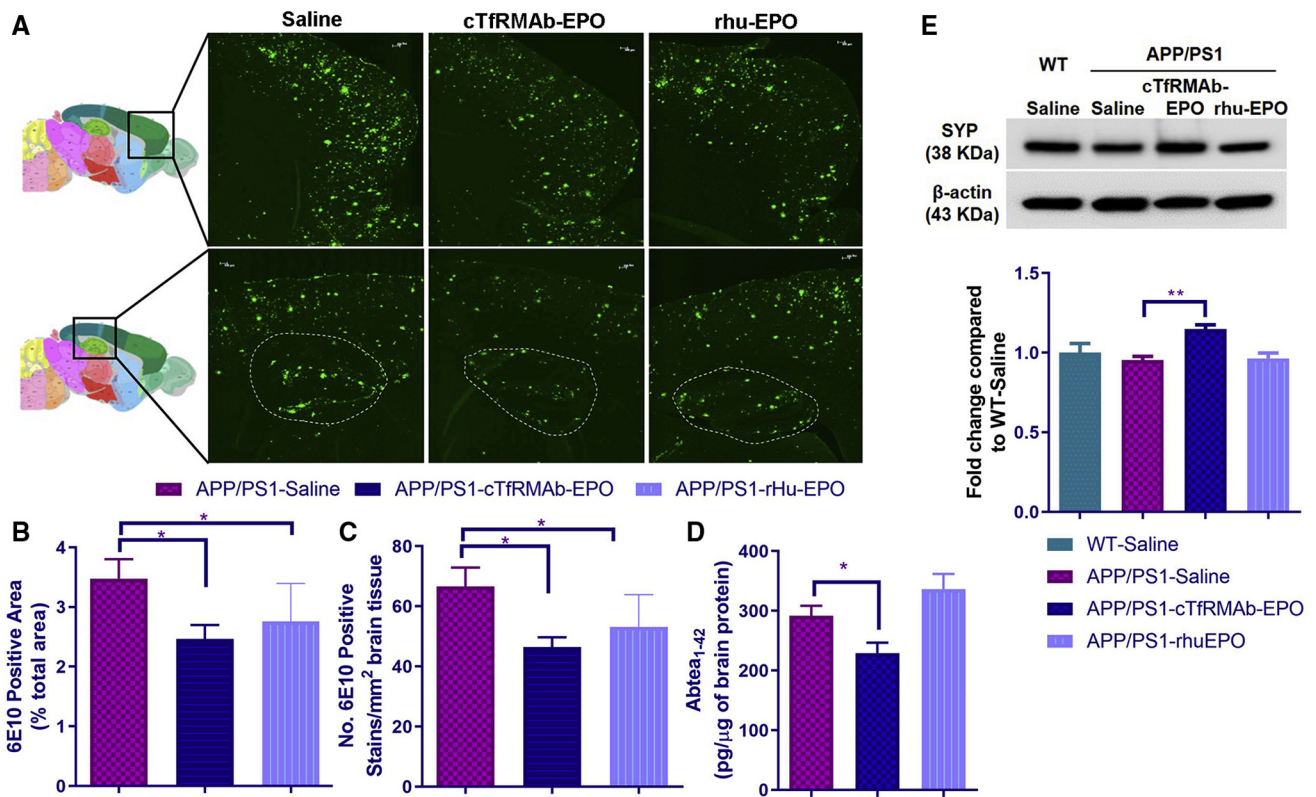


Fig. 3. Effect of cTfRMAb-EPO and rhu-EPO on A $\beta$  load and neuronal function. Representative images of 6E10-positive A $\beta$  peptide in the brains of saline-, cTfRMAb-EPO-, and rhu-EPO-treated APP/PS1 mice. The hippocampus is outlined in white (A). Significant reduction in the brain 6E10-positive A $\beta$  peptide area (B) and number (C) in cTfRMAb-EPO- and rhu-EPO-treated APP/PS1 mice compared with saline-treated APP/PS1 controls. A significant reduction in insoluble A $\beta$ (1–42) level in cTfRMAb-EPO-treated mice compared with the APP/PS1-saline controls (D). Synaptophysin protein level was significantly increased in the cTfRMAb-EPO-treated APP/PS1 mice compared with the saline-treated APP/PS1 controls (E). Data are presented as mean  $\pm$  SEM of 7–11 mice per group. Kruskal-Wallis with Dunn's multiple comparisons test or one-way ANOVA with Holm-Sidak's post hoc test was used to compare to the APP/PS1-saline group. \* $P < .05$ , \*\* $P < .01$ . Abbreviations: A $\beta$ , amyloid beta; EPO, erythropoietin.

after treatment initiation (2 weeks after stopping treatment) (Table 1).

### 3.2. Behavior analysis

Locomotion and exploration at 6 weeks were expressed as percentage of baseline to highlight treatment effects (Fig. 2A–C). Mean speed and total distance was significantly lower, whereas resting time was significantly higher, in APP/PS1-rhu-EPO mice compared to WT-saline mice (Fig. 2A–C and E). No significant change of locomotion and exploration was observed in APP/PS1-saline and APP/PS1-cTfRMAb-EPO mice. Time in the center, an indicator of anxiety-like behavior, was not significantly different between the experimental groups (Fig. 2D–E).

The present study was not powered for memory assessment, and we therefore calculated a composite memory score to determine the effect of treatment on overall memory impairment. The APP/PS1-saline mice had a lower composite z-score compared with the WT-saline mice, and the z-score value reached borderline significance ( $P = .076$ ; Fig. 2F). Chronic treatment of APP/PS1 mice with rhu-EPO worsened performance on the memory tests as seen by the significantly

lower composite z-score ( $P < .01$ ; Fig. 2F), while the composite z-score value of APP/PS1-cTfRMAb-EPO mice did not differ from WT-saline mice.

### 3.3. A $\beta$ load and synaptic function

There was a significant reduction in the 6E10-positive A $\beta$ -peptide area in APP/PS1-rhu-EPO mice (21% decrease;  $P < .05$ ) and APP/PS1-cTfRMAb-EPO mice (29% decrease;  $P < .05$ ) compared with APP/PS1-saline mice (Fig. 3A–B). Similarly, the number of 6E10-positive A $\beta$ -peptide stains was also significantly lower in APP/PS1-rhu-EPO mice (20% decrease;  $P < .05$ ) and APP/PS1-cTfRMAb-EPO mice (30% decrease;  $P < .05$ ) compared with APP/PS1-saline controls (Fig. 3A and C). The APP/PS1-cTfRMAb-EPO mice had lower levels of brain A $\beta$ (1–42) compared with APP/PS1-saline mice (25% reduction;  $P < .05$ ). Notably, no reduction of brain A $\beta$ (1–42) was observed in APP/PS1-rhu-EPO mice (Fig. 3D). Synaptic function was assessed by measuring synaptophysin, a presynaptic vesicle protein, in the brain. As shown in Fig. 3E, synaptophysin level was significantly higher in APP/PS1-cTfRMAb-EPO mice than

APP/PS1-saline controls. No significant difference was observed in synaptophysin levels in the brains of APP/PS1-rhu-EPO mice compared with APP/PS1-saline controls.

**4. Discussion**

Here we elucidate the hematologic safety of a brain-penetrable EPO analog, cTfRMab-EPO, after chronic dosing in the APP/PS1 mice. We show that cTfRMab-EPO transiently increases reticulocytes at 4 weeks, followed by normalization of reticulocytes at 6 and 8 weeks, whereas all other RBC indices remain unchanged compared to the WT-saline controls (Table 1). Chronic treatment with equimolar doses of rhu-EPO on the other hand induces significant changes in hematologic parameters that are associated with a significant reduction in exploration and locomotion. Furthermore, rhu-EPO treatment did not improve memory impairment in APP/PS1 mice (Fig. 2). Both the brain-penetrable EPO and rhu-EPO significantly reduced diffuse Aβ-immunopositivity (Fig. 3B–C). The brain-penetrable EPO, however, selectively reduced brain Aβ(1-42) and increased synaptophysin expression, and these therapeutic effects were not observed in the APP/PS1-rhu-EPO mice (Fig. 3D and E).

EPO binds to EPO receptors (EPOR) on the surface of the erythroid progenitor cells to stimulate erythropoiesis in the bone marrow [18,19] and is clinically used to treat anemia [20,21]. Besides its role in erythropoiesis, EPO is a neurotrophin and offers therapeutic benefits in mouse models of neural diseases [22], and in the past decade, numerous studies have reported the protective effects of chronic rhu-EPO dosing in experimental AD [23–28]. Given the role of EPO in proliferation and differentiation of erythroid progenitor cells, hematopoietic side effects are the major concern associated with chronic rhu-EPO use; however, only a small number of studies report the hematopoietic effects of chronic rhu-EPO use in experimental AD [24]. In the present study, RBC indices were transiently elevated at 4 weeks followed by a decline at 8 weeks in the APP/PS1-rhu-EPO mice compared with WT-saline controls (Table 1). Notably, reticulocytes were significantly reduced at 4 weeks in APP/PS1-rhu-EPO mice. The initial change in RBC indices at 4 weeks is consistent with a study by Armand-Ugon et al. [24], wherein 4-week treatment, 3 days a week, with rhu-EPO significantly increased hematocrit but reduced reticulocytes in APP/PS1 mice. The decline in the RBC indices at 8 weeks is, on the other hand, consistent with underlying anemia. Anemia may result from increased splenic RBC sequestration and RBC destruction or reduced erythropoiesis in the bone marrow [29,30]. In the present study, rhu-EPO treatment was not associated with spleen enlargement (Fig. 1), and splenic RBC-sequestration as a cause of anemia in the

**Table 2**  
White blood cell indices of mice treated with saline, cTfRMab-EPO, or rhu-EPO for 6 weeks followed by 2-week recovery period

Parameter	4 weeks			6 weeks			8 weeks					
	WT	APP/PS1	APP/PS1	WT	APP/PS1	APP/PS1	WT	APP/PS1	APP/PS1			
	saline	saline	cTfRMAB-EPO	saline	saline	rhu-EPO	saline	saline	rhu-EPO			
WBC (10 <sup>3</sup> /μL)	4.7 ± 0.6	6.9 ± 1.5	8.0 ± 0.2	7.4 ± 0.8	5.8 ± 1.8	7.3 ± 1.7	6.5 ± 0.50	8.4 ± 1.6	3.9 ± 0.34	4.6 ± 0.9	4.9 ± 0.3	5.2 ± 1.1
PMN (%)	21.7 ± 2.9	19.7 ± 5.8	20.0 ± 1.2	10.7 ± 0.9	21.9 ± 2.8	26.7 ± 4.2	19.8 ± 2.1	19.2 ± 1.6	34.4 ± 6.4	33.8 ± 5.3	24.3 ± 3.2	21.8 ± 2.6
Lymph (%)	70.7 ± 2.4	72.7 ± 7.9	69.3 ± 2.0	82.3 ± 0.9	68.5 ± 3.4	61.9 ± 6.4	71.1 ± 3.2	71.7 ± 2.5	54.3 ± 7.3	54.8 ± 7.1	68.3 ± 3.7	71.5 ± 2.4
Mono (%)	6.3 ± 1.7	5.3 ± 2.3	9.0 ± 2.5	5.7 ± 0.3	6.1 ± 0.6	8.7 ± 2.3	6.6 ± 1.0	5.8 ± 1.0	7.7 ± 1.4	7.3 ± 2.1	5.0 ± 0.1	4.1 ± 0.4
Eos (%)	1.3 ± 0.3	2.3 ± 0.3	1.7 ± 0.3	1.3 ± 0.3	2.9 ± 1.2	2.6 ± 0.2	2.1 ± 0.3	3.1 ± 0.1	3.1 ± 0.9	3.7 ± 0.4	2.1 ± 0.4	2.5 ± 0.4
Baso (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	0.2 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.2 ± 0.0

Abbreviations: WBC, white blood cell; PMN, polymorphonuclear neutrophils; Lymph, lymphocytes; Mono, monocytes; Eos, eosinophils; Baso, basophils; WT, wild-type. Data are presented as mean ± SEM.

Table 3  
Summary of safety profile and therapeutic effects of chronic treatment with cTfRMAB-EPO and rhu-EPO in APP/PS1 mice

Parameter	4 weeks		6 weeks		8 weeks	
	cTfRMAB-EPO	rhu-EPO	cTfRMAB-EPO	rhu-EPO	cTfRMAB-EPO	rhu-EPO
<b>Hematology</b>						
Hematocrit	↔	↑	↔	↔	↔	↓
RBC	↔	↑	↔	↔	↔	↓
Retic	↑	↓	↔	↓	↔	↓
MCV	↔	↓	↔	↓	↔	↓
Hemoglobin	↔	↑	↔	↔	↔	↓
WBC	↔	↔	↔	↔	↔	↔
<b>Behavior</b>						
Distance and speed	————	————	————	————	↔	↓
Resting time	————	————	————	————	↔	↑
Memory	————	————	————	————	↔	↓
<b>Therapeutic effects (compared to APP/PS1-saline)</b>						
Total amyloid load	————	————	————	————	↓	↓
Aβ(1-42)	————	————	————	————	↓	↔
Synaptophysin	————	————	————	————	↑	↔

Abbreviations: Aβ, amyloid beta; MCV, mean corpuscular volume; RBC, red blood cell; WBC, white blood cell; WT, wild-type. All comparisons were made with WT-saline unless indicated otherwise.

APP/PS1-rhu-EPO mice was therefore ruled out. Alternatively, EPO resistance, which is characterized by anemia along with low reticulocytosis in the presence of elevated circulating EPO levels, may be responsible for low RBC indices in the APP/PS1-rhu-EPO mice at 8 weeks [31,32]. Moreover, desensitization of EPOR due to continuous receptor activation has also been reported [33]. rhu-EPO dose used herein (0.6 mg/kg equivalent to ~60,000 IU/kg) is 12- to 24-fold higher than doses used in experimental AD, and many-fold higher than doses used for anemia ( $\leq 500$  IU/kg) [34]. It is therefore conceivable that prolonged EPOR activation due to chronic high-dose rhu-EPO treatment herein results in EPOR desensitization and/or EPO resistance leading to reduced erythropoietic activity in the bone marrow. Notably, rhu-EPO-associated reticulocyte reduction is observed even at low chronic rhu-EPO doses (25  $\mu$ g/kg) in APP/PS1 mice [24].

In contrast to the negative hematologic effects of rhu-EPO, cTfRMAB-EPO did not alter the hematologic profile of APP/PS1 mice in the present study. Reticulocytes were however significantly elevated in the APP/PS1-cTfRMAB-EPO mice at 4 weeks (Table 1). Our recent work showed reticulocyte suppression within 24 hours after a single 3 mg/kg dose of cTfRMAB-EPO [35], and this finding is consistent with other studies showing reticulocyte suppression with high-affinity TfRMAB treatment [36]. Seven days after injection of high doses of the high-affinity TfRMAB, reticulocytes were however significantly higher compared to controls [36]. Taken together, reticulocytes are suppressed at 24 hours but elevated weeks after TfRMAB treatment and this increase in reticulocytes may be a response to the acute reticulocyte

suppression associated with high-affinity TfRMAB treatment. These results collectively suggest that the acute reticulocyte suppression associated with cTfRMAB-EPO is a transient response that gradually resolves with chronic treatment.

The stark differences in the hematologic profiles of APP/PS1-cTfRMAB-EPO and APP/PS1-rhu-EPO mice reported herein can be attributed to the differences in the plasma clearance of these two EPO analogs. cTfRMAB-EPO has a faster plasma clearance compared with rhu-EPO owing to the cTfRMAB domain which results in TfR-mediated peripheral cTfRMAB-EPO clearance [7,11,37]. The cTfRMAB thus drives the EPO into the brain to reduce the hematologic side effects that are observed with chronic rhu-EPO treatment.

Previous work shows that chronic 3-week treatment with cTfRMAB-EPO results in low-titer anti-drug antibody formation [12]. We however did not observe any immune response, for example, injection-related reactions, including profound lethargy, spastic movements, scruffy hunched appearance, and reddish-brown urine, or an increase in white blood cell count with either cTfRMAB-EPO or rhu-EPO treatment (Table 2). Notably, injection-related reactions have been reported after a single high dose injection of a humanized high-affinity TfRMAB in mice [36]. The absence of an immune response herein is consistent with our previous work [13].

We observed age effect on general locomotion and exploration of novel environment for the WT and APP/PS1 mice (Fig. 2), which is consistent with previous findings showing reduced locomotion with age in mice [38]. However, treatment effect on locomotion and exploration



was significant only for the APP/PS1-rhu-EPO mice, and rhu-EPO significantly reduced locomotion and exploration compared to WT-saline mice. This reduction in exploration may be attributed to lethargy due to underlying reduction in RBC indices in these APP/PS1-rhu-EPO mice.

We recently showed that cTfRMAb-EPO reduces A $\beta$  load, microglial activation, and neuronal loss in APP/PS1 mice. However, how these effects compare with rhu-EPO treatment was unknown [13]. Here, we found a significant reduction in 6E10-positive A $\beta$  load in APP/PS1-cTfRMAb-EPO and APP/PS1-rhu-EPO mice. The A $\beta$ -lowering effect of rhu-EPO seen herein is consistent with other studies [23,24] and is attributed to a reduction in the receptor for advanced glycation end products (RAGE) [23]. RAGE is involved in the blood-to-brain transport of plasma A $\beta$  and its expression is abnormally increased in AD [39]. Given the luminal expression of RAGE, both the brain-penetrable EPO and rhu-EPO can alter its expression to produce A $\beta$ -lowering effects seen herein. The A $\beta$  load measured using 6E10 MAb in the present study stains all A $\beta$  forms including the precursor form [40]; however, A $\beta$ (1–42) is the more pathologic, aggregation prone, form of A $\beta$  [41]. Brain A $\beta$ (1–42) levels were significantly lower only in APP/PS1-cTfRMAb-EPO mice but not in APP/PS1-rhu-EPO mice, demonstrating the selective effect of the brain-penetrating EPO on this pathogenic form of A $\beta$ . Similarly, the brain-penetrable EPO significantly increased the expression of synaptophysin confirming our previous findings of increased synaptic function in APP/PS1-cTfRMAb-EPO mice [13]. Notably, this protective effect was not observed in APP/PS1-rhu-EPO mice. Similarly, no improvement in memory function was observed in APP/PS1-rhu-EPO mice (Fig. 2). The overall memory function in APP/PS1-cTfRMAb-EPO mice was comparable to WT-saline mice and is consistent with our previous finding of improvement in memory function with cTfRMAb-EPO in experimental AD [13].

In summary, the present study confirms the therapeutic potential and elucidates the hematologic safety of cTfRMAb-EPO compared to rhu-EPO in the APP/PS1 mice (Table 3). These beneficial effects are attributed to the cTfRMAb domain of cTfRMAb-EPO which (1) drives EPO into the brain to offer neuroprotection and (2) increases the plasma clearance to reduce the hematopoietic side effects associated with long-term EPO treatment. The cTfRMAb-EPO is thus a CNS-penetrant EPO analog, which is devoid of the hematologic side effects that are observed with chronic high-dose rhu-EPO use.

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### RESEARCH IN CONTEXT

1. Systematic review: We performed a literature search using PubMed for literature related to the development of erythropoietin (EPO) for Alzheimer's disease (AD). Collectively, research shows that EPO is a promising neurotherapeutic for AD; however, low blood-brain barrier penetration and negative hematopoietic effects have limited its therapeutic development for AD.
2. Interpretation: A blood-brain barrier-penetrable EPO analog was engineered by fusing EPO to a chimeric monoclonal antibody targeting the mouse transferrin receptor (cTfRMAb), which (1) ferries EPO into the brain and (2) enables faster peripheral clearance of EPO to reduce hematopoietic effects. Herein, we demonstrate that chronic treatment with cTfRMAb-EPO results in better hematologic, behavioral/cognitive, and therapeutic indices compared with recombinant human EPO in APP/PS1 mice.
3. Future directions: These results set the stage for future studies in other experimental models of AD, dose-response studies, and combination-treatment studies with other agents, to further the development of cTfRMAb-EPO for AD.

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