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BRIEF REPORT

Regulation of platelet lifespan in the presence and absence of thrombopoietin signaling

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Essentials

- We examined platelet survival in models of absent or enhanced thrombopoietin (TPO) signaling.
- Platelet lifespan is normal in transgenic mice with chronically enhanced TPO signaling.
- Mpl deficiency does not negatively affect platelet lifespan in the absence of thrombocytopenia.
- We conclude that TPO and its receptor Mpl are dispensable for platelet survival in adult mice.

Summary. *Background:* It is well established that thrombopoietin (TPO), acting via its receptor Mpl, is the major cytokine regulator of platelet biogenesis. The primary mechanism by which TPO signaling stimulates thrombopoiesis is via stimulation of Mpl-expressing hematopoietic progenitors; Mpl on megakaryocytes and platelets acts to control the amount of TPO available. TPO could potentially reduce platelet and/or megakaryocyte apoptosis, and therefore increase the platelet count. However, the effect of TPO receptor signaling on platelet survival is unresolved. *Methods and results:* Here, we investigated platelet survival in mouse models of absent or enhanced TPO signaling. In the absence of thrombocytopenia, *Mpl* deficiency did not negatively influence platelet lifespan, and nor was platelet survival affected in transgenic mice with chronically increased TPO signaling. *Conclusions:* We conclude that TPO and its receptor Mpl are dispensable for platelet survival in adult mice.

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Introduction

Thrombopoietin (TPO) is the major regulator of platelet production [1]. Mpl on megakaryocytes and platelets acts to control the amount of TPO available to hematopoietic stem and progenitor cell populations [2]. Recent work has demonstrated that the survival of megakaryocytes and platelets is controlled by programmed cell death, i.e. apoptosis [3]. Both cell types possess a classic Bak-mediated and Bax-mediated intrinsic, mitochondrial, apoptosis pathway that must be restrained for them to develop and survive. Bcl-x_L is the critical prosurvival protein required to maintain platelet *in vivo* survival [4,5], whereas megakaryocyte survival is dependent on both Bcl-x_L and Mcl-1 [6–8]. Previous studies have revealed that TPO signaling regulates Bcl-x_L and Mcl-1 expression in megakaryoblastic cells through the Jak–Stat5 and extracellular signal-regulated kinase pathways [8,9]. TPO could therefore potentially increase Bcl-x_L expression and reduce megakaryocyte and/or platelet apoptosis, and thus increase the platelet count. Conversely, one might expect an absence of TPO signaling to result in less Bcl-x_L, resulting in compromised platelet survival. In mice, loss of Bak and Bax almost doubles the platelet lifespan [7], and overexpression of prosurvival BCL-2 extends platelet survival [10]. However, the effect of TPO receptor signaling on platelet survival is unresolved. TPO-mimetic drugs are currently used to boost platelet production in conditions of thrombocytopenia, including immune thrombocytopenia (ITP). Recently, it was reported that administration of TPO-mimetic drugs in ITP patients transiently altered platelets' apoptotic profile [11], whereas loss of *Mpl* in mice is associated with thrombocytopenia [1] and a reduced platelet lifespan [12]. Here, we investigated platelet survival in mouse models of absent or enhanced TPO signaling.

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Materials and methods

Mice

Tpo^{Tg} [13], $Bcl\text{-}x^{Plt20/Plt20}$ [4], $Mpl^{-/-}$ [1], $c\text{-}Myb^{Plt4/Plt4}$ [14] and DsRed [15] mice have been previously described. All strains were backcrossed onto the C57BL/6 background for at least 10 generations. Male and female mice were aged 7–10 weeks, or as otherwise stated. All animal experiments complied with the regulatory standards of, and were approved by, the Walter and Eliza Hall Institute Animal Ethics Committee.

Hematology

Automated cell counts were performed on blood collected by cardiac puncture or from the retro-orbital plexus into EDTA-coated tubes (Sarstedt, Ingle Farm, South Australia, Australia), with an Advia 2120 hematological analyzer (Siemens, Munich Germany). Reticulated platelets were stained with thiazole orange [16]. Megakaryocytes were counted manually in sections of the sternum and spleen stained with hematoxylin and eosin, with a minimum of 10 high-power fields (× 200) being analyzed. Serum TPO levels were measured with the mouse TPO Quantikine ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Megakaryocyte ploidy

Bone marrow was harvested from femurs into citrate–adenosine–theophylline in Hank's balanced salt solution, and megakaryocyte ploidy was studied by staining with a CD41–fluorescein isothiocyanate mAb and propidium iodide, as described previously [7].

Platelet preparation

Blood was obtained by cardiac puncture into a 0.1 volume of Aster–Jandl anticoagulant (85 mM sodium citrate, 69 mM citric acid, and 20 mg mL^{-1} glucose, pH 4.6) [16]. Platelet-rich plasma (PRP) was obtained by centrifugation at 125 × *g* for 8 min, followed by centrifugation of the supernatant buffy coat at 125 × *g* for 8 min. Platelets were washed by two sequential centrifugations at 860 × *g* for 5 min in 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, and 12.5 mM sucrose (pH 6.0) (buffer A). The pellet was resuspended in 10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 10 mM glucose, and 0.5 mM $NaHCO_3$ (pH 7.4) (buffer B).

Mpl expression determined with flow cytometry

Purified platelets were stained with a biotinylated rat anti-mouse Mpl mAb (clone AMM2; Immuno-Biological Laboratories, Minneapolis, MN, USA) for 30 min, followed by streptavidin– allophycocyanin (APC) for 40 min, and washed by centrifugation (5 min, 860 × *g*) in buffer A containing 1 μg mL^{-1} prostaglandin E_1.

Platelet turnover studies

Wild-type (WT) mice were injected intravenously with 0.15 μg g^{-1} body weight of X488 (Emfret Analytics, Eibelstadt, Germany), a rat-derived IgG against the platelet CD42c receptor conjugated with Dylight488, and platelet lifespan was measured as described previously [16]. The amount of X488 injected was adjusted on the basis of platelet counts, in order to achieve similar degrees of *in vivo* platelet surface labeling (micrograms of X488 injected as compared with WT: Tpo^{Tg}, two-fold; $Bcl\text{-}x^{Plt20/Plt20}$, 0.5–fold; $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$, one-fold; $c\text{-}Myb^{Plt4/Plt4}$, two-fold; $Mpl^{-/-}$, 0.5-fold; and $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$, two-fold). At various time points, whole blood was isolated from the tail vein (3–4 μL) and mixed with anticoagulant (25 μL of Aster–Jandl; 100 μL of buffer B). Care was taken to apply pressure and to ensure that no additional bleeding occurred. Diluted PRP was isolated after centrifugation for 8 min at 125 × *g*. Platelets were stained with fluorescently conjugated rat anti-mouse CD41 (clone MWReg30; BD, Franklin Lakes, NY, USA) for 20 min at room temperature. Platelets were identified in PRP as being $CD41^+$ by flow cytometry, and the proportion of $X488^+$ platelets remaining at each time point was assessed.

Platelet transfusions were performed with platelets purified from WT, Tpo^{Tg}, $c\text{-}Myb^{Plt4/Plt4}$, $Mpl^{-/-}$ and $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$ mice, labeled with 5-chloromethyl fluorescein diacetate (CMFDA) (Invitrogen Life Technologies, Carlsbad, CA, USA) after pooling of platelets of the same genotype [17]. The washed platelet pellets were resuspended in a small volume of phosphate-buffered saline, and platelet counts were determined with Sphero blank calibration beads (Spherotech, Lake Forest, IL, USA) on a FACSCalibur flow cytometer. Platelet counts were adjusted to 3×10^8 cells mL^{-1} (Fig. 1F), 1×10^9 cells mL^{-1} (Fig. 3D), or 1×10^8 cells mL^{-1} (Fig. 3E), and 200 μL was transfused per recipient WT mouse. Platelets were identified in PRP as being CD41–phycoerythrin-positive, and the proportion of $CMFDA^+$ platelets remaining at each time point was assessed. One hundred per cent was set on the basis of 1-h or 3-h time points post-transfusion. In some experiments, DsRed WT platelets were purified, and 150 μL of 3.5×10^8 cells mL^{-1} was transfused into WT and $Mpl^{-/-}$ recipient mice (Fig. 2C). Platelets were identified in PRP as being $CD41\text{–}APC^+$.

SDS-PAGE and western blot analysis

Platelets were lysed in NP40 lysis buffer, and proteins were separated on 4–12% Bis-Tris gels (NuPAGE; Invitrogen Life Technologies) under reducing conditions, transferred onto Immobilon-P membranes (Merck Millipore, Billerica, MA,

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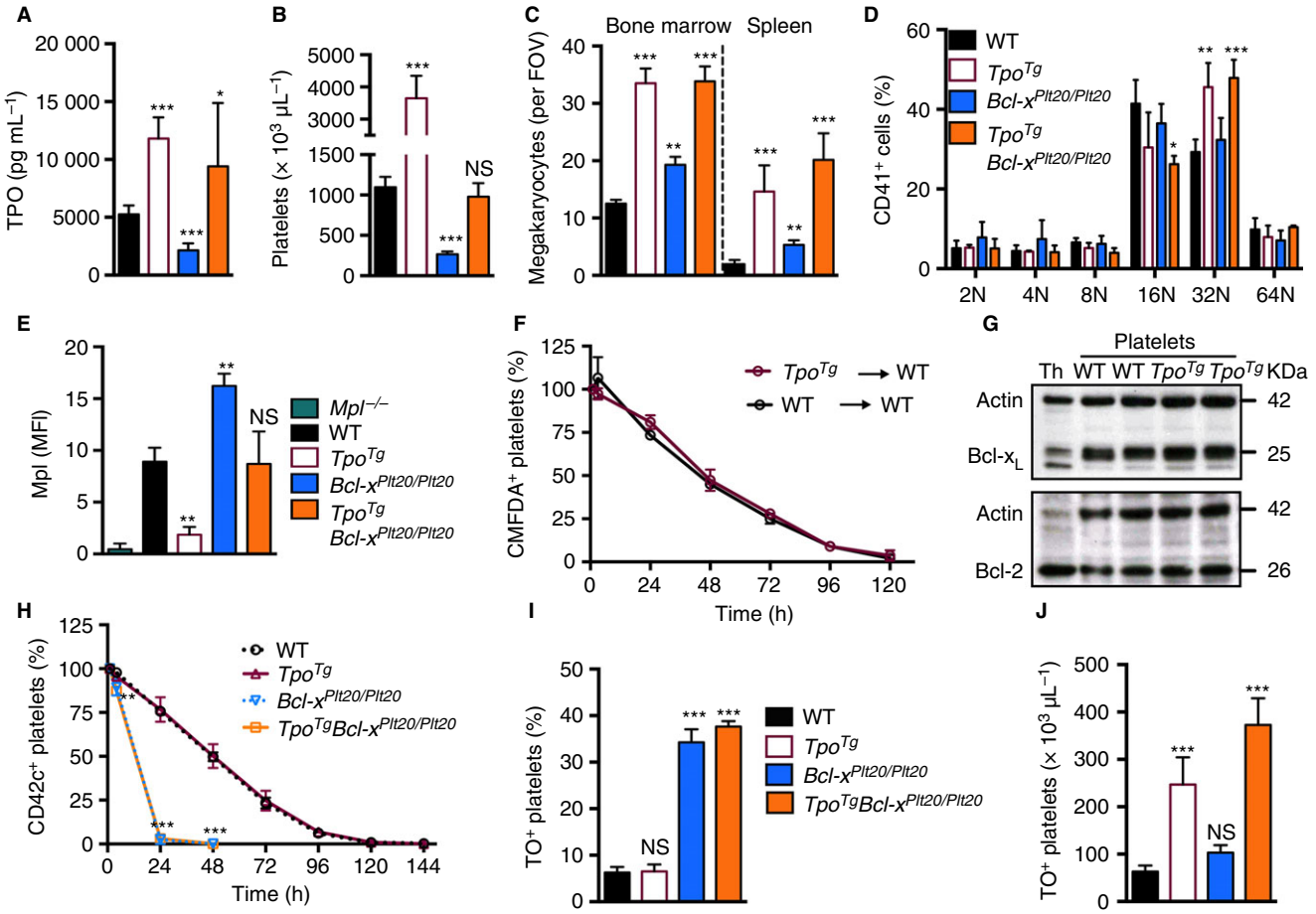

Fig. 1. Chronic thrombopoietin (TPO) stimulation normalizes platelet counts in thrombocytopenic mice by increased thrombopoiesis. (A) Serum TPO levels in 7–10-week-old wild-type (WT) (n = 9), Tpo^{Tg} (n = 5), $Bcl\text{-}x^{Plt20/Plt20}$ (n = 8) and $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ (n = 4) mice. (B) Peripheral blood platelet counts in WT, Tpo^{Tg}, $Bcl\text{-}x^{Plt20/Plt20}$ and $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ mice at 7–10 weeks of age; n = 28–29 mice per genotype. (C) Morphologically recognizable megakaryocytes enumerated from microscopic examination of hematoxylin and eosin-stained sternum and spleen sections from WT, Tpo^{Tg}, $Bcl\text{-}x^{Plt20/Plt20}$ and $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ mice; n = 4 mice per genotype. (D) Ploidy distribution profile of $CD41^+$ bone marrow cells from WT, Tpo^{Tg}, $Bcl\text{-}x^{Plt20/Plt20}$ and $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ mice, determined by flow cytometry; n = 4 mice per genotype. (E) Platelet Mpl expression determined by flow cytometry; n = 3–5 mice per genotype. $Mpl^{-/-}$ platelets were included as a negative control. (F) Platelet survival of transfused 5-chloromethyl fluorescein diacetate $(CMFDA)^+$ Tpo^{Tg} platelets. Platelets were purified from WT and Tpo^{Tg} mice, labeled with CMFDA, and injected intravenously. into WT recipient mice. Time 0 (100%) was set at 1 h post-injection; n = 3 recipient mice per group. (G) Representative western blot of protein lysates from WT and Tpo^{Tg} platelets. Each lane represents platelets from an individual mouse. Thymocytes (Th) were used as a positive control for Bcl-x_L and Bcl-2 protein expression. Probing for actin was used as a control for protein loading. (H) Platelet survival in WT, Tpo^{Tg}, $Bcl\text{-}x^{Plt20/Plt20}$ and $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ mice. Platelets were labeled via intravenous injection of DyLight488-conjugated anti-CD42c. Time 0 (100%) was set at 1 h post-injection; n = 3–4 mice per genotype. (I, J) Proportion (I) and absolute number (J) of reticulated thiazole orange $(TO)^+$ platelets in WT, Tpo^{Tg}, $Bcl\text{-}x^{Plt20/Plt20}$ and $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ mice; n = 4 mice per genotype. Data are presented as mean ± standard deviation. Data are compared with those for the WT. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$. FOV, field of view; MFI, mean fluorescence intensity; NS, not significant.

USA), immunoblotted with various antibodies, and then incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies and enhanced chemiluminescence. Antibodies: mouse anti-BCL-2 (clone 7; recognizes mouse, rat, chicken, dog, and human); anti-Bcl-xL (clone 44; recognizes mouse, rat, and human) (BD); and anti-actin–HRP (Santa Cruz Biotechnology, Dallas, TX, USA).

Statistical analyses

Statistical significance between two treatment groups was analyzed by use of an unpaired Student's t-test with two-tailed P-values. One-way ANOVA with Dunnett's multiple comparison test was applied where appropriate (GRAPHPAD PRISM Version 6.0g). Data are presented as mean ± standard deviation.

Results and discussion

Chronic elevated TPO stimulation does not affect platelet lifespan

To investigate the effect of elevated TPO stimulation on platelet lifespan, transgenic mice expressing elevated levels

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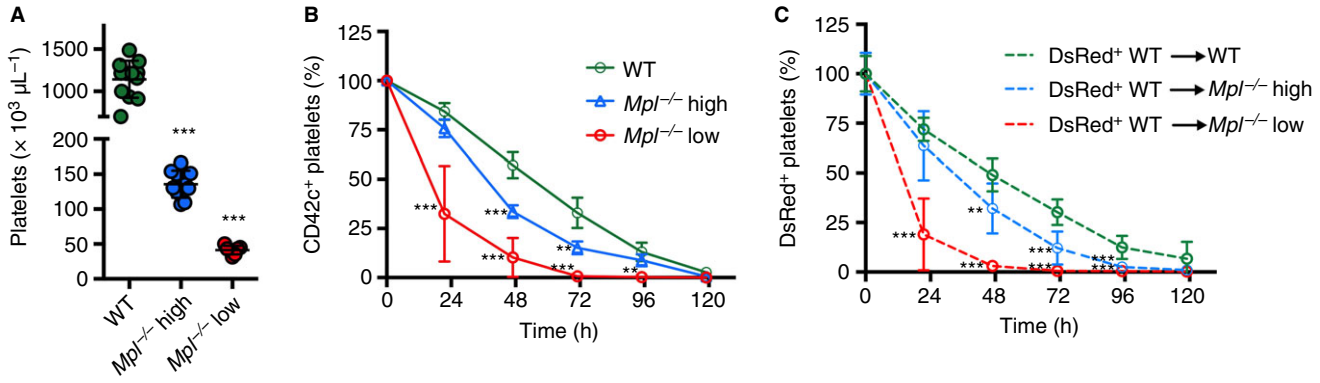

Fig. 2. Wild-type (WT) platelets have a reduced lifespan in $Mpl^{-/-}$ mice. (A) Peripheral blood platelet counts in WT and $Mpl^{-/-}$ mice at 6–10 weeks of age. $Mpl^{-/-}$ platelets were separated into two groups on the basis of platelet counts, defined as $Mpl^{-/-}$ high and low. Each symbol represents an individual mouse. (B) Platelet survival in WT, $Mpl^{-/-}$ high and $Mpl^{-/-}$ low mice. Platelets were labeled via intravenous injection of DyLight488-conjugated anti-CD42c. Time 0 (100%) was set at 1 h post-injection. WT, $n = 8$ mice; $Mpl^{-/-}$ high, $n = 3$ mice; and $Mpl^{-/-}$ low, $n = 3$ mice. (C) WT DsRed platelets, purified from DsRed mice, were transfused, and their survival time was determined (dashed lines) in recipient WT ($n = 7$), $Mpl^{-/-}$ high ($n = 7$) and $Mpl^{-/-}$ low ($n = 5$) mice. Time 0 (100%) was set at 1 h post-injection. Data are presented as mean ± standard deviation. Data are compared with those for the WT. $^{**}P < 0.005$; $^{***}P < 0.001$.

of circulating TPO, i.e. Tpo^{Tg} mice [13] (Fig. 1A), were crossed with mice exhibiting an intrinsic reduction in platelet lifespan owing to a mutation in prosurvival Bcl-x_L (Plt20) [4]. Chronic elevated TPO stimulation (Fig. 1A) normalized platelet counts in $Bcl\text{-}x^{Plt20/Plt20}$ mice (Fig. 1B), owing to an increase in megakaryocyte number and increased ploidy in bone marrow and spleen (Fig. 1C, D). Furthermore, platelet Mpl expression returned to normal (Fig. 1E). To determine whether platelets produced in an environment with elevated TPO levels had acquired intrinsic changes affecting their survival, platelets fluorescently labeled with CMFDA [17] from Tpo^{Tg} and WT mice were transfused into WT recipients. We found that neither platelet survival (Fig. 1F) nor the expression of prosurvival Bcl-x_L and Bcl-2 proteins in purified platelets from Tpo^{Tg} and WT mice (Fig. 1G) were significantly different, indicating that the effects of excess TPO on platelet survival were negligible. Similarly, platelet survival was not extended in Tpo^{Tg} versus WT mice or in $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ versus $Bcl\text{-}x^{Plt20/Plt20}$ mice (Fig. 1H), as assessed by Dylight488–CD42c *in vivo* labeling of platelets. Furthermore, $Tpo^{Tg}Bcl\text{-}x^{Plt20/Plt20}$ and $Bcl\text{-}x^{Plt20/Plt20}$ mice showed equally high proportions of young reticulated platelets (Fig. 1I,J), consistent with their platelets being short-lived [4]. Taken together, these results indicate that chronic elevated TPO stimulation enhances megakaryopoiesis, but does not affect platelet lifespan. These data are consistent with a recent clinical study assessing the long-term effects of TPO receptor agonists on the apoptotic profile of platelets in patients with chronic ITP [11].

WT platelets have a reduced lifespan in $Mpl^{-/-}$ mice

To examine the effect on platelet survival of an absence of Mpl signaling, we labeled platelets *in vivo* with Dylight488–CD42c in WT and $Mpl^{-/-}$ mice. As previously described, thrombocytopenic $Mpl^{-/-}$ mice show reduced hematopoietic progenitor cell and megakaryocyte numbers [1], and can be divided into two groups on the basis of low and extremely low platelet counts [12]. Figure 2A shows these groups: $Mpl^{-/-}$ 'high' ([136 ± 19] × 10^3 µL^{-1}, ~ 12% of WT) and $Mpl^{-/-}$ 'low' ([41 ± 6] × 10^3 µL^{-1}, ~ 3.5% of WT), relative to WT platelet counts ([1143 ± 218] × 10^3 µL^{-1}). Similarly to Coupland *et al.* [12], we found reduced platelet survival in both $Mpl^{-/-}$ groups (Fig. 2B), but this was exacerbated in the $Mpl^{-/-}$ low group. It was previously concluded that compromised vascular integrity resulting in microscopic bleeding caused reduced platelet and erythrocyte lifespans in $Mpl^{-/-}$ low mice [12]. Nevertheless, as no effect on erythrocyte lifespan was reported in the $Mpl^{-/-}$ high group, although these mice showed a reduced platelet lifespan, it was speculated that an intrinsic TPO effect also existed [12]. To determine potential environmental effects on platelet survival in $Mpl^{-/-}$ mice, we transfused them with fluorescent DsRed [15] WT platelets. Interestingly, the survival of transfused WT platelets (DsRed$^+$) followed the same pattern, with increasingly reduced platelet survival as compared with the WT, $Mpl^{-/-}$ high and $Mpl^{-/-}$ low host environments (Fig. 2C). This finding prompted us to investigate the survival of $Mpl^{-/-}$ platelets in the absence of thrombocytopenia.

$Mpl^{-/-}$ platelets survive normally in the absence of thrombocytopenia

$c\text{-}Myb^{Plt4/Plt4}$ mice show thrombocytosis ([3936 ± 618] × 10^3 µL^{-1}) independently of TPO signaling, driven by a point mutation in the transcription factor *c-Myb* [14]. This effect is caused by increases in megakaryocyte progenitor and megakaryocyte cell numbers. The use of $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$ mice (platelet count of [4662 ± 851] × 10^3 µL^{-1} [14]) allowed us to determine the platelet

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in vivo lifespan in the absence of TPO signaling independently of thrombocytopenia. First, we confirmed the lack of platelet Mpl expression on $Mpl^{-/-}$-$c\text{-}Myb^{Plt4/Plt4}$ platelets by flow cytometry (Fig. 3A). In agreement with de Graaf *et al.* [13], serum TPO levels were increased in $Mpl^{-/-}$ mice (Fig. 3B). Dylight488–CD42c was injected intravenously into WT, $c\text{-}Myb^{Plt4/Plt4}$, $Mpl^{-/-}$ and $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$ mice, and platelet lifespan was assessed (Fig. 3C). As expected, platelet lifespan was normal in $c\text{-}Myb^{Plt4/Plt4}$ mice, and was significantly reduced in $Mpl^{-/-}$ mice ($Mpl^{-/-}$ high, $[169 \pm 32] \times 10^3$ μL^{-1}; ~ 15% of normal). Strikingly, survival of *Mpl*-deficient platelets was not compromised in $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$ mice (Fig. 3C). This result was further confirmed after transfusion of CMFDA-labeled $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$ and $Mpl^{-/-}$ platelets into WT recipient mice (Fig. 3D,E), and is in agreement with our recent study of *Mpl*-deficient platelet survival in $Mpl^{PF4Cre/PF4Cre}$ mice [2].

Our results demonstrate that, in the absence of thrombocytopenia, *Mpl* deficiency does not reduce platelet lifespan. Hence, platelet Mpl expression is not required for the regulation of platelet survival. It is not surprising that the severe thrombocytopenia present in the $Mpl^{-/-}$ low mice led to increased consumption because of microscopic bleeding, as described previously [12]. However, in light of the recent Morowski [18] study, no bleeding would have been expected in the $Mpl^{-/-}$ high group, but these mice showed a reduced platelet lifespan via a mechanism speculated to be intrinsic [12]. However, as host and transfused WT platelets both had a reduced circulation time in $Mpl^{-/-}$ high and low mice, we cannot exclude extrinsic effects from a potentially proinflammatory environment [19] or reduced functional capacity of $Mpl^{-/-}$ platelets as contributory factors in a setting of thrombocytopenia. A detailed quantitative analysis of intrinsic and extrinsic effects on platelet lifespan, production rates and peripheral blood platelet counts with mathematical modeling could help to resolve this problem [20–22].

A recent clinical study found that platelets from ITP patients treated with TPO receptor agonists were less prone to apoptosis when treated with the Bcl-x_L inhibitor

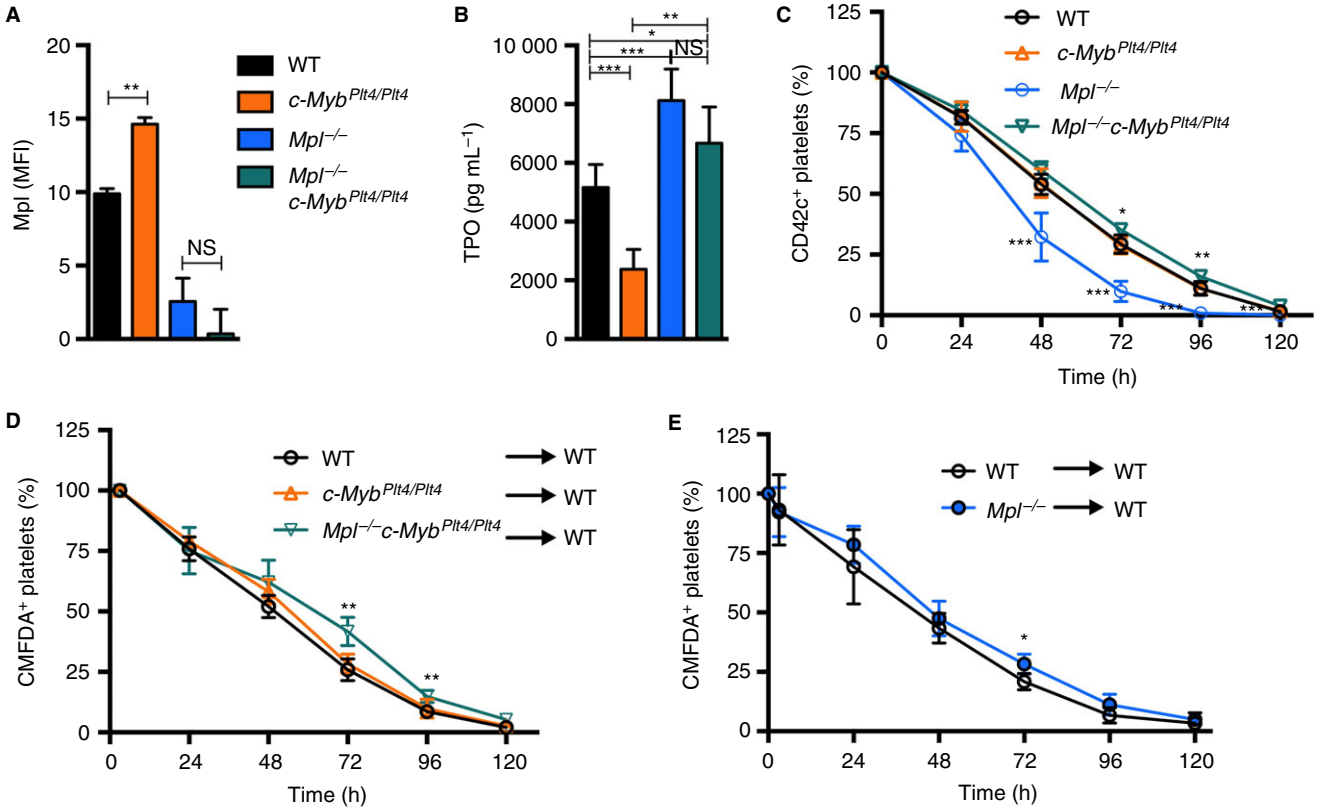

Fig. 3. $Mpl^{-/-}$ platelets survive normally in the absence of thrombocytopenia. (A) Platelet Mpl surface expression determined by flow cytometry; $n = 3$ mice per genotype. $Mpl^{-/-}$ platelets were included as a negative control. (B) Serum thrombopoietin (TPO) levels in 7–10-week-old mice. WT, $n = 8$ mice; $c\text{-}Myb^{Plt4/Plt4}$, $n = 3$ mice; $Mpl^{-/-}$, $n = 5$ mice; and $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$, $n = 3$ mice. (C) Platelet survival in wild-type (WT), $c\text{-}Myb^{Plt4/Plt4}$, $Mpl^{-/-}$ and $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$ mice. Platelets were labeled via intravenous injection of DyLight488-conjugated anti-CD42c. Time 0 (100%) was set at 3 h post-injection; $n = 4$–6 mice per genotype. (D) Platelets were purified from WT, $c\text{-}Myb^{Plt4/Plt4}$ and $Mpl^{-/-}c\text{-}Myb^{Plt4/Plt4}$ mice, labeled with 5-chloromethyl fluorescein diacetate (CMFDA), and injected intravenously into WT recipient mice. Time 0 (100%) was set at 3 h post-injection; $n = 3$–6 recipient mice per group. (E) Platelets were purified from WT and $Mpl^{-/-}$ mice, labeled with CMFDA, and injected intravenously into WT recipient mice. Time 0 (100%) was set at 1 h post-injection; $n = 4$ recipient mice per group. Data are presented as mean ± standard deviation. Data are compared with those for the WT, if not otherwise indicated. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$. MFI, mean fluorescence intensity; NS, not significant.

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ABT-737 after the first week, but returned to baseline after a longer period of administration (2 weeks) [11]. Consistent with this study, chronically increased TPO signaling in mice did not affect platelet survival.

Taking these findings together, we conclude that TPO receptor signaling is dispensable for platelet survival in adult mice.

Addendum

M. Lebois and E. C. Josefsson designed and performed research, analyzed data, and wrote the paper. M. R. Dowling designed research and analyzed data. P. Gangatirkar performed research and analyzed data. W. S. Alexander, B. T. Kile, and P. D. Hodgkin provided reagents, designed research, and revised the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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