Protein Kinase C δ Deficiency Enhances Megakaryopoiesis and Recovery From Thrombocytopenia

John C. Kostyak, Dheeraj Bhavanasi, Elisabeta Liverani, Steven E. McKenzie, Satya P. Kunapuli

Objective—We previously determined that protein kinase C δ (PKCδ) regulates platelet function. However, the function of PKCδ in megakaryopoiesis is unknown.

Approach and Results—Using PKCδ−/− and wild-type littermate mice, we found that deficiency of PKCδ caused an increase in white blood cells and platelet counts, as well as in bone marrow and splenic megakaryocytes (P<0.05). Additionally, the megakaryocyte number and DNA content were enhanced in PKCδ−/− mouse bone marrow after culturing with exogenous thrombopoietin compared with wild-type (P<0.05). Importantly, thrombopoietin-induced signaling was also altered with PKCδ deletion because both extracellular signal-regulated kinase and Akt308 phosphorylation were heightened in PKCδ−/− megakaryocytes compared with wild-type. Finally, PKCδ−/− mice recovered faster and had a heightened rebound thrombocytosis after thrombocytopenic challenge.

Conclusions—These data suggest that PKCδ is an important megakaryopoietic protein, which regulates signaling induced by thrombopoietin and represents a potential therapeutic target. (Arterioscler Thromb Vasc Biol. 2014;34:2579-2585.)

Key Words: blood platelets ▪ megakaryocytes ▪ protein kinase C ▪ thrombopoietin

Megakaryocytes, which arise chiefly in the bone marrow, are responsible for producing platelets. They accomplish this task by projecting proplatelets into bone marrow sinusoidal vessels. However, the megakaryocyte must undergo a unique maturation process to develop the resources necessary to perform this function. The hallmark of this maturation process is a modified cell cycle known as endomitosis, in which the megakaryocyte replicates its DNA but does not divide. Instead, through repeated rounds of endomitosis, a large polyploid cell capable of producing platelets is formed. The primary regulator of this process is the cytokine thrombopoietin.

Thrombopoietin engagement of its receptor, c-Mpl, leads to activation of several signaling pathways, including Janus kinase 2, signal transducers and activators of transcription, phosphoinositide 3-kinase, Akt, and mitogen-activated protein kinase. Inhibition of any of these signaling pathways will inhibit megakaryocyte differentiation. Recently, negative regulators of thrombopoietin-mediated signaling have been identified including focal adhesion kinase, the Src-family kinase Lyn, and calcium- and integrin-binding protein 1. Together, a complex view of thrombopoietin-mediated signaling begins to emerge. Although the role of PKCs in phorbol ester-mediated endomitosis is defined, little regarding the function of PKCs in primary megakaryopoiesis has been reported.

PKCs are a family of serine/threonine protein kinases that have a wide range of functions. There are three classes of PKCs; conventional PKCs (α, β1, β2, and γ) which require diacylglycerol and Ca²⁺ for activation, novel PKCs (δ, ε, η, and θ) which require only diacylglycerol for activation, and atypical PKCs (ζ, ι/λ) which require phospholipids for their activation. We have previously reported that PKCδ is important for platelet function. In platelets, PKCδ negatively regulates GPVI-mediated dense granule secretion, but positively regulates PAR4-mediated dense granule secretion. In megakaryocytes, PKCδ transcript and protein expression are enhanced compared with CD34+ progenitor cells. However, the function of PKCδ in primary megakaryocyte differentiation has not yet been elucidated.

To define the role of PKCδ in megakaryopoiesis, we used a murine knockout model. We determined that PKCδ−/− mice have more platelets and megakaryocytes than wild-type (WT) littermate control mice. Furthermore, the culture of PKCδ−/− bone marrow using exogenous thrombopoietin produced a greater number of and more highly polyploid megakaryocytes than WT littermate bone marrow. This may be because of enhanced thrombopoietin-mediated signaling because PKCδ−/− megakaryocytes have elevated extracellular signal-regulated kinase (ERK1/2) and Akt308 phosphorylation compared with WT littermate megakaryocytes after stimulation with thrombopoietin. Importantly, in an in vivo experiment PKCδ−/− mice...
recovered faster than WT littermate mice from thrombocytopenia. These data strongly suggest that PKCδ is an endogenous negative regulator of thrombopoietin-mediated megakaryocyte differentiation.

**Methods**

Methods are available in the online-only Data Supplement.

**Results**

**PKCδ Expression is Elevated During Megakaryocyte Differentiation**

Previous reports show that PKCδ mRNA expression and PKCδ protein expression are enhanced in human megakaryocytes compared with progenitor cells.25,26 To determine whether PKCδ is also important for mouse megakaryopoiesis, we performed similar experiments using mouse progenitors and megakaryocytes. First, we isolated bone marrow progenitor cells from WT mice and incubated them in Iscove’s Modified Dulbecco’s Medium either containing 50 ng/mL recombinant mouse thrombopoietin, or not. After 7 days of culture we analyzed the DNA content of megakaryocytes from both Tpo− (thrombopoietin) and Tpo+ cultures. We were able to generate a sizeable quantity of mature megakaryocytes from Tpo+ cultures (Figure 1A). Therefore, we harvested progenitor cells (0 days) and cells after 3, 5, and 7 days of culture to analyze PKCδ protein expression throughout these time periods. Interestingly, we found that PKCδ protein expression was elevated after 3 days of culture and continued to increase up to 7 days of culture (Figure 1B), suggesting that PKCδ expression increases during megakaryocyte differentiation. Additionally, we compared PKCδ expression in megakaryocytes purified using a discontinuous BSA gradient after culturing bone marrow, and found that PKCδ expression was much greater in megakaryocytes than bone marrow mononuclear cells (Figure 1C). These data suggest that PKCδ protein expression is enhanced during megakaryocyte differentiation and that PKCδ may play an important role in megakaryopoiesis.

**PKCδ Deletion Enhances Circulating Platelet Counts**

Using a hemavet blood analyzer and blood drawn via cardiac puncture, we determined that PKCδ−/− mice have more circulating platelets than WT littermate mice (Table 1). The platelet volume was not altered, and there was a slight but not significant decrease in the plasma thrombopoietin concentration with PKCδ deficiency (Table 1). Additionally, PKCδ−/− mice also had enhanced lymphoproliferation (Table 1). These data suggest that deletion of PKCδ in mice causes increased circulating platelet counts.

**PKCδ−/− Mice have Enhanced Bone Marrow Megakaryocyte Proliferation and Platelet Production**

An increased platelet count suggests that megakaryopoiesis may be altered in PKCδ deficient mice. To determine that

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>SHIP-1</td>
<td>SH2 domain-containing inositol phosphatase-1</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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**Figure 1.** Protein kinase C δ (PKCδ) protein expression is enhanced during megakaryocyte differentiation. **A,** CD34+ progenitor cells were cultured with or without 50 ng/mL recombinant mouse thrombopoietin for 7 days and the DNA content was quantified via flow cytometry. **B,** CD34+ cells cultured in the presence of 50 ng/mL exogenous thrombopoietin for 0, 3, 5, or 7 days were lysed and resolved via SDS-PAGE, then probed for PKCδ. **C,** Mouse mononuclear bone marrow cells and megakaryocytes were lysed and resolved using SDS-PAGE, then probed for PKCδ. Extracellular signal-regulated kinase was used to assess loading in both A and B.
PKCδ deficiency did not result in changes of other PKC isoform protein expression, we performed western blot analysis of PKCδ−/− and WT littermate control megakaryocyte lysate. We found that all PKC isoforms tested (α, β, ε, θ) had equal expression in PKCδ−/− compared with WT control (data not shown). Therefore, we analyzed the bone marrow megakaryocyte number and DNA content in PKCδ−/− and WT littermate mice using flow cytometry. Interestingly, we did not observe any differences in the DNA content between PKCδ−/− and WT littermate megakaryocytes isolated directly from bone marrow (Figure 2A). However, we found that PKCδ−/− mice contain more bone marrow megakaryocytes than WT littermate mice (Figure 2B). This is in agreement with data presented in Table 1, which shows that PKCδ−/− mice produce more platelets than WT littermate mice. An increased circulating platelet number suggests that platelet production may be altered in PKCδ−/− mice. Therefore, we quantified the number of “new” platelets using thiazole orange to identify reticulated platelets. We observed that the increase in the platelet number with PKCδ deletion was indeed because of increased platelet production, as PKCδ−/− mice had significantly more reticulated platelets per blood cell than WT littermate mice (Figure 2C). Furthermore, we determined platelet clearance in PKCδ−/− mice and found that it was unchanged compared with WT littermate control mice, suggesting that enhanced circulating platelet counts observed in PKCδ−/− mice are because of enhanced platelet production (Figure 2D).

**Splenic Megakaryopoiesis is Enhanced in PKCδ-Deficient Mice**

Platelet production is altered in PKCδ−/− mice; so we investigated megakaryocyte production in the spleen, which is another site of hematopoiesis in mice. We found that PKCδ−/− mice had much larger spleens than WT littermate mice (Figure 3A and 3B), which is in agreement with a previous report.27 This could be because of enhanced lymphocyte production as previously reported. However, megakaryopoiesis in PKCδ−/− mouse spleens has not yet been described. Therefore, we sectioned PKCδ−/− and WT littermate mouse spleens, and stained with Hematoxylin & Eosin. We determined that PKCδ−/− mouse spleens contained more megakaryocytes than WT littermate spleens, which is in agreement with our data collected in bone marrow (Figure 3C and 3D).

**Deletion of PKCδ Enhances the Megakaryocyte DNA Content and Proliferation After Bone Marrow Culture**

To further characterize the role of PKCδ in megakaryopoiesis, we cultured bone marrow from PKCδ−/− and WT littermate mice in the presence of 50 ng/mL exogenous thrombopoietin. Using flow cytometry, we observed an increase in the proportion of highly polyploid megakaryocytes present in PKCδ−/− bone marrow cultures compared with WT littermate cultures (Figure 4A). To quantify this difference we compared the proportion of mature (16 N+) megakaryocytes to that of immature (2 N to 8 N) megakaryocytes in both WT littermate and PKCδ−/− mouse bone marrow. We found that the majority of megakaryocytes in WT littermate cultures had a DNA content equal to or <8 N. However, the majority of megakaryocytes in PKCδ−/− cultures had a DNA content >8 N (Figure 4B), suggesting that PKCδ deletion enhances megakaryocyte differentiation. Additionally, we quantified the number of megakaryocytes compared with nucleated cells in each culture and found a higher proportion of megakaryocytes in the PKCδ−/− bone marrow cultures compared with cultures from WT littermate bone marrow (Figure 4C).

PKCδ regulates apoptosis in a variety of cells. One reason for the enhanced number of megakaryocytes observed in PKCδ−/− mouse bone marrow and spleen could be the resistance to apoptosis because of PKCδ deficiency. Therefore, we assessed apoptosis in WT and PKCδ−/− megakaryocytes via

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**Table 1. Blood Cell Counts and Plasma Thrombopoietin Levels in PKCδ−/− and WT Littermate Mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>PKCδ−/−</th>
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<tr>
<td>Platelets, 10^3/μL</td>
<td>579±49</td>
<td>1100±229*</td>
</tr>
<tr>
<td>Mean platelet volume, fl.</td>
<td>4.949±0.058</td>
<td>5.359±0.174</td>
</tr>
<tr>
<td>Plasma thrombopoietin, pg/mL</td>
<td>532.5±78.97</td>
<td>377.5±117.95</td>
</tr>
<tr>
<td>White blood cells, 10^3/μL</td>
<td>5.77±0.19</td>
<td>5.35±0.87</td>
</tr>
<tr>
<td>Neutrophils, 10^3/μL</td>
<td>0.55±0.08</td>
<td>0.64±0.03</td>
</tr>
<tr>
<td>Lymphocytes, 10^3/μL</td>
<td>4.03±0.19</td>
<td>7.16±0.91</td>
</tr>
<tr>
<td>Monocytes, 10^3/μL</td>
<td>0.18±0.02</td>
<td>0.39±0.05</td>
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*P<0.05, n=8. PKCδ−/− indicates protein kinase C δ; and WT, wild-type.

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**Figure 2. Bone marrow megakaryocyte number and platelet production are enhanced with protein kinase C δ (PKCδ deletion).**

A. Megakaryocyte DNA content from wild-type (WT) littermate and PKCδ−/− mice was quantified using flow cytometry. B. Bone marrow megakaryocyte number from WT littermate and PKCδ−/− mice was quantified via flow cytometry and expressed per nucleated bone marrow cell. C. Anti-coagulated blood from WT littermate and PKCδ−/− mice was stained with thiazole orange. The total number of thiazole orange positive platelets was quantified and expressed per 50 000 total blood cells. D. Platelet clearance was assessed via flow cytometry. The number of bixin positive platelets was quantified and expressed as percent original.

*P<0.05, n=6.
We did not observe any differences between WT and PKCδ-/- cells (data not shown) suggesting that PKCδ does not regulate megakaryocyte apoptosis and that the reason for enhanced megakaryopoiesis in PKCδ-/- mice must be something else.

**PKCδ is a Negative Regulator of Thrombopoietin-Induced Signaling**

Because we noted that megakaryopoiesis was heightened with PKCδ deficiency we aimed to determine whether or not PKCδ regulates thrombopoietin-induced signaling. After 5 days culture, megakaryocytes from WT littermate and PKCδ-/- bone marrow were purified, and treated with 50 ng/mL thrombopoietin to induce signaling. SDS-PAGE of megakaryocyte lysates revealed that both ERK and Akt308 phosphorylation were significantly enhanced in PKCδ-/- mouse megakaryocytes (Figure 5A and 5B). ERK and Akt are known regulators of thrombopoietin-mediated signaling. To verify that the observed increase in ERK and Akt phosphorylation is not attributed to altered cell surface expression of the thrombopoietin receptor, we analyzed the surface expression of c-Mpl on PKCδ-/- and WT littermate control megakaryocytes via flow cytometry. We determined that PKCδ deficiency does not alter the surface expression of c-Mpl (data not shown). Therefore, these data suggest that PKCδ is an integral negative regulator of thrombopoietin-induced signaling.

**Proplatelet Production is Unaltered with PKCδ-/- Deficiency**

To determine whether or not PKCδ influences proplatelet production, we plated purified bone marrow megakaryocytes on immobilized fibrinogen and quantified megakaryocytes that

**Figure 3.** Splenic megakaryopoiesis is enhanced with protein kinase C δ (PKCδ) deficiency. **A,** Representative spleens from wild-type (WT) littermate and PKCδ-/- mice. **B,** Spleen weight was quantified and expressed per gram body weight (n=8). **C,** Representative spleen sections from WT littermates and PKCδ-/- mice, stained with Hematoxylin & Eosin. White arrows indicate megakaryocytes. Images were taken using a Nikon Eclipse E600 microscope with a Nikon DMX1200 camera. **D,** Megakaryocytes were quantified from each section and expressed per high power field (n=5). *P<0.05.

**Figure 4.** Megakaryocyte DNA content and number are enhanced with protein kinase C δ (PKCδ) deletion after the culture of bone marrow cells. **A,** Representative histograms of the megakaryocyte DNA content from wild-type (WT) littermate and PKCδ-/- mouse bone marrow cultures. **B,** Quantification of the megakaryocyte DNA content expressed as immature (2 N to 8 N) megakaryocytes and mature (16 N+) megakaryocytes. **C,** Quantification of the megakaryocyte number from WT littermate and PKCδ-/- bone marrow cultures, expressed per nucleated cell. *P<0.05, n=6.
produced proplatelets. We observed no differences in the frequency of proplatelet-producing megakaryocytes (Figure 6A and 6B). Additionally, proplatelet-producing megakaryocytes from PKC\(\delta\)-/- bone marrow seemed to be structurally similar to proplatelet-producing megakaryocytes from littermate control bone marrow. These data suggest that PKC\(\delta\) does not influence proplatelet production.

Recovery From Immune-Mediated Thrombocytopenia is Enhanced in PKC\(\delta\)-/- Mice
To elucidate whether or not the alterations in megakaryopoiesis, we observed with PKC\(\delta\) deficiency in vitro were relevant in vivo, we created an immune-mediated thrombocytopenia and monitored recovery. Platelet counts in both WT littermate and PKC\(\delta\)-/- mice were greatly reduced 24 hours after injection of a mouse anti-CD41 antibody (Figure 6C). However, the PKC\(\delta\)-/- mice recovered platelet counts at a higher rate than WT littermate mice such that PKC\(\delta\)-/- mice were fully recovered after 3 days, whereas WT littermate mice took \(\approx\)4 days. Additionally, rebound thrombocytosis was greatly enhanced in PKC\(\delta\)-/- mice compared with WT littermates. These data suggest that PKC\(\delta\) regulates megakaryopoiesis and platelet production in vivo and establishes itself as an important therapeutic target for thrombocytopenia.

Discussion
In this report, we demonstrate that PKC\(\delta\) is an important regulator of thrombopoietin-mediated megakaryopoiesis. We demonstrated that PKC\(\delta\) protein expression is enhanced during megakaryocyte differentiation, suggesting that it may be a regulatory protein. Deletion of PKC\(\delta\) in mice caused an increase in platelet production possibly because of enhanced megakaryocyte production in the bone marrow and spleen. Furthermore, the culture of mouse bone marrow produced heightened megakaryocyte differentiation and proliferation in PKC\(\delta\)-/- tissue compared with WT littermates. We also revealed that PKC\(\delta\) could be a regulator of thrombopoietin-mediated signaling as phosphorylated ERK1/2 and Akt308 were enhanced with PKC\(\delta\) deficiency. Finally, recovery from immune-mediated thrombocytopenia was enhanced in...
PKCδ−/− mice compared with WT littermate mice. These data strongly suggest that PKCδ is an important regulator of bone marrow megakaryopoiesis.

PKCδ protein expression is enhanced during megakaryocyte differentiation, and PKCδ protein expression is greater in megakaryocytes than mononuclear bone marrow cells. These data are in agreement with previous reports regarding both mRNA expression and protein expression in megakaryocytes compared with CD34+ progenitor cells. A possible explanation for increased PKCδ protein expression in megakaryocytes could be to ensure adequate PKCδ expression in platelets because platelets have limited ability to undergo protein synthesis. Furthermore, we have previously reported that PKCδ is an important regulator of platelet function. However, the data presented in this report suggest that PKCδ protein expression is also tied to megakaryocyte differentiation. Therefore, we theorize that PKCδ regulates both megakaryopoiesis and platelet function after it is transported to the platelet from the megakaryocyte.

Deletion of PKCδ in mice causes enhanced platelet production. Likewise, PKCδ−/− mice have more circulating platelets than WT littermate mice. An increased platelet count can arise from either increased platelet production or inhibited platelet destruction. In this report, we show that platelet production is greatly increased with PKCδ deletion, whereas platelet clearance remains unaltered, suggesting that the reason for enhanced platelet counts is because of heightened platelet production. Additionally, we report here that the megakaryocyte number is enhanced in the bone marrow of PKCδ−/− mice, further implying that enhanced platelet production is responsible for the increase in platelet counts observed in PKCδ−/− mice.

PKCδ may be a negative regulator of thrombopoietin-dependent signaling as pERK1/2 and pAkt308 are elevated in PKCδ−/− megakaryocytes after treatment with exogenous thrombopoietin. Furthermore, the bone marrow megakaryocyte number is enhanced in PKCδ−/− mice, and the megakaryocyte DNA content and number are enhanced after the culture of PKCδ−/− bone marrow. We have previously reported that PKCδ is a negative regulator of GPVI-mediated signaling via interactions with the Src-family kinase Lyn, and SH2 domain-containing inositol phosphatase-1 (SHIP-1). Interestingly, Lyn, which is activated by FAK downstream of c-Mpl, is also a negative regulator of thrombopoietin-mediated signaling. In Lyn−/− megakaryocytes, thrombopoietin-mediated ERK and Akt phosphorylation are enhanced, whereas SHIP-1 tyrosine phosphorylation is depressed. Therefore, it is possible that PKCδ is activated by Lyn downstream of c-Mpl and is responsible for phosphorylating and activating SHIP-1, which is known to regulate phosphoinositide 3-kinase. Additionally, knockdown of SHIP-1 in erythroleukemia cells caused enhanced phosphoinositide 3-kinase and ERK1/2 phosphorylation compared with control. Together, these data suggest that PKCδ regulates thrombopoietin-dependent megakaryocyte differentiation perhaps through interactions with both Lyn and SHIP-1. It would be most interesting to determine whether PKCδ interacts with these 2 proteins after stimulation of megakaryocytes with thrombopoietin.

In addition to enhanced megakaryopoiesis and platelet production, PKCδ−/− mice have enhanced lymphoproliferation, which is in agreement with a previous report. Interestingly, 2 PKCδ deficient patients have recently been described. The patients have enhanced lymphoproliferation, lymphadenopathy, and splenomegaly similar to the PKCδ−/− mouse. Unfortunately, no data regarding platelets or megakaryocytes have been reported. However, the similarities between the mouse and human data suggest that alterations in megakaryopoiesis in humans with PKCδ deficiency are likely. Any such data would further cement PKCδ as a potential therapeutic target.

We demonstrated that PKCδ is an important mediator of megakaryocyte differentiation and subsequent platelet production. PKCδ−/− mice have more platelets and megakaryocytes than WT littermate mice and they recover faster after immune thrombocytopenia. PKCδ may regulate megakaryopoiesis by inhibiting thrombopoietin-mediated signaling because both ERK and Akt308 phosphorylation were enhanced with PKCδ deficiency. Therefore, PKCδ may represent an important therapeutic target for thrombocytopenia.

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Disclosures

None.

References

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Significance

Thrombocytopenia or low platelet count is a condition that can be caused by reduced production of platelets, increased destruction of platelets, or by the use of certain therapeutics. Current treatment of thrombocytopenia includes platelet transfusion. However, there are significant risks associated with this approach, and the donor pool cannot currently support demand. Thrombopoietin therapy is also used and can be effective. However, another therapeutic that could synergize with thrombopoietin therapy could be most useful. In this report we evaluate the role of protein kinase C δ in megakaryopoiesis. We demonstrate that protein kinase C δ deficiency augments thrombopoietin-mediated signaling, megakaryopoiesis and thrombopoiesis. Importantly, we note that protein kinase C δ deficiency enhances recovery from thrombocytopenia in mice. These data suggest that protein kinase C δ could be a therapeutic target, and because specific inhibitors of protein kinase C δ are currently in production testing this hypothesis may soon be possible.
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**Antibodies and reagents**

All reagents were purchased from Sigma-Aldrich unless otherwise noted. Cell culture reagents including, Iscove’s Modified Dulbecco’s Medium (IMDM), heat-inactivated fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were purchased from Thermo Fisher (Pittsburgh, PA). Polyclonal antibodies against phospho-ERK1/2, ERK1/2, Phospho-Akt308, were purchased from Cell Signaling Technologies (Danvers, MA). A polyclonal antibody against PKCδ was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). FITC-labeled anti-CD41 antibody and a purified anti-mouse CD41 antibody were purchased from BD Biosciences (San Jose, CA). Annexin-V-PE apoptosis detection kit was purchased from BD Biosciences.

**Animals**

PKCδ−/− mice were a generous gift from Dr. Keiko Nakayama (Division of developmental genetics, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan). All procedures were approved by the Temple University Institutional Animal Care and Use Committee. All mice are on a C57BL/6 background.

**Blood cell enumeration**

Blood was collected from PKCδ−/−, and age-matched littermate mice via cardiac puncture. Anticoagulated blood was then analyzed using a Hemavet (Drew Scientific Inc., Waterbury, CT).

**Plasma TPO Quantification**

Plasma was isolated from anti-coagulated blood from PKCδ−/− and WT control mice via centrifugation. Plasma Tpo concentrations were quantified using a Quantikine ELISA mouse thrombopoietin kit from R&D Systems (Minneapolis, MN) as per manufacturers instructions.

**Platelet production assay**

Platelet production was assessed as previously described with minor modifications [1]. Briefly, 1 µL of blood was added to 60 µL of 2 mM EDTA in PBS containing 10 µg/mL Thiazole Orange and incubated from 30 minutes at room temperature. Each sample was then fixed in 1 mL of 1% formalin for 15 minutes at room temperature and analyzed by flow cytometry using a FACScalibur (BD Biosciences). The thiazole orange positive platelet population was quantified.

**Platelet clearance**

An assay to determine murine platelet clearance was performed as previously described [1]. Briefly, PKCδ−/− and WT littermate control mice 10-12 weeks of age were injected with 35mg/g bodyweight sulfo-NHS-biotin (Pierce) via tail vein. Blood was collected via submandibular bleed 3 hours after tail vein injection and then every 24 hours for 4 days. Blood was diluted 20X in PBS and incubated with thiazole orange and streptavidin-PE antibody (BD Biosciences) for 30 minutes. After fixation in 1% formalin the platelets were analyzed via flow cytometry with appropriate color compensation.

**Megakaryocyte DNA content**

Quantification of megakaryocyte DNA was assessed as previously described [1, 2]. Briefly, bone marrow femurs and tibiae of WT littermate and PKCδ−/− mice was flushed into IMDM then passed through a 22-gauge needle to create a single-cell suspension. After centrifugation at 340 X g for 3 minutes, then cells were resuspended in ACK buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA, pH 7.4), and incubated for 5 minutes at room temperature to lyse the red cells. The cells were then washed in PBS and resuspended in either IMDM containing P/S, FBS, and 50 ng/mL Tpo (for bone marrow culture), or PBS containing 2 mM EDTA (for baseline megakaryocyte ploidy analysis). To analyze megakaryocyte DNA content 2 X 10⁶ cells (directly
from bone marrow or after 5 days culture) were resuspended in PBS containing 2mM EDTA and labeled with a FITC-conjugated CD-41 antibody for 30 minutes at 4°C. The cells were washed twice in PBS containing 2mM EDTA and fixed in 0.5% formalin for 20 minutes at room temperature. The cells were washed again and permeabilized with 70% methanol for 60 minutes at 4°C. After washing, the cells were then treated with 10mg/mL RNAase A for 30 minutes at 37°C to digest the RNA. The cells were then treated with 10 µg/mL propidium iodide and analyzed using an LSRII flow cytometer and FACSDiva software (BD Biosciences).

**Hematoxylin and eosin staining**
Spleens from WT littermate and PKCδ−/− mice were embedded in paraffin and sectioned at a thickness of 5µm and stained with H&E as previously described. Megakaryocytes were identified by size and nuclear morphology, and were quantified in each high power field (HPF). At least 5 HPF’s were analyzed per section and at least 5 sections per mouse.

**Western blotting**
To analyze PKCδ expression, bone marrow cells were cultured (as described in *megakaryocyte DNA content*). Megakaryocytes were separated from mononuclear cells using a discontinuous BSA gradient (0%, 1.5%, 3%), washed in PBS, and lysed using an NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, and 1:100 Halt® (Thermo Fisher) protease and phosphatase cocktail)15,26. Progenitor (CD34+) cells were isolated from bone marrow using an Easy Sep mouse hematopoietic progenitor cell enrichment kit from Stem Cell technologies (Vancouver, BC) according to the manufacturers instructions then either lysed using NP-40 lysis buffer, or cultured (as described in *megakaryocyte DNA content*) for up to 7 days. Megakaryocytes were then isolated using a discontinuous BSA gradient, and lysed for Western blot analysis. Samples were then boiled in 2X Laemmeli buffer for 5 minutes prior to resolving via SDS-PAGE. The proteins were then transferred to PVDF membrane and probed for PKCδ (1:2000), or ERK1/2 (1:1000).
To analyze the effect of PKCδ deletion in Tpo-induced signaling, megakaryocytes from WT littermate and PKCδ−/− bone marrow cultures (as described in *megakaryocyte DNA content*) were purified using a discontinuous BSA gradient, and serum-starved overnight at 37°C. Megakaryocytes were then washed and resuspended in PBS prior to the addition of 50ng/mL Tpo or ddH2O. The cells were then incubated at 37°C for 10 minutes and immediately lysed in NP-40 lysis buffer. Each sample was then boiled in 2X Laemmeli buffer for 5 minutes prior to resolving via SDS-PAGE. The proteins were then transferred to PVDF membrane and probed for phospho-ERK1/2 (1:2000), or phospho-Akt308 (1:1000). After imaging the membranes were stripped using Western Restore (Thermo Fisher) and probed for either Akt (1:1000) or ERK1/2 (1:1000) to assess loading. All Western blots are representative of at least 3 independent experiments.

**C-Mpl surface expression**
Bone marrow from PKCδ−/− and littermate control WT mice was cultured to expand the megakaryocyte population. Megakaryocytes were purified using a discontinuous BSA gradient and either labeled with anti-C-Mpl antibody from Abcam (Cambridge, MA) or exposed to control IgG. After secondary antibody labeling and fixation the megakaryocytes were analyzed by flow cytometry.

**Analysis of megakaryocyte apoptosis**
Megakaryocytes from WT littermate and PKCδ−/− mice were isolated from bone marrow cultures using a discontinuous BSA gradient. The megakaryocytes were then dual labeled with annexin V and 7AAD using a PE annexin V apoptosis detection kit (BD Biosciences) according to
manufacturer's instructions. The cells were analyzed using an LSRII flow cytometer and FACSdiva software.

**Proplatelet production assay**

Bone marrow megakaryocytes from PKCδ<sup>−/−</sup> and WT littermate control mice were expanded and purified using a discontinuous BSA gradient. Megakaryocytes were then plated on 100mg/mL immobilized fibrinogen and allowed to incubate overnight at 37°C. Images were then taken using a Nikon eclipse TE300 at 200X magnification. The number of proplatelet producing megakaryocytes were enumerated and expressed as a percent on total megakaryocytes.

**Immune-induced thrombocytopenia**

Thrombocytopenia was induced in 10-12 week old WT littermate and PKCδ<sup>−/−</sup> mice as previously described [1]. Briefly, mice were injected IP with 50 µg/kg anti-mouse CD41 antibody following a baseline blood cell count. Following the injection, blood was drawn via submandibular puncture every 24 hours for 5 days and platelets were enumerated [3].
References

