Thrombocytosis as a Response to High Interleukin-6 Levels in cGMP-Dependent Protein Kinase I Mutant Mice

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Objective—The purpose of this study was to investigate the influence of cGMP-dependent kinase I (cGKI) on platelet production.

Approach and Results—We used hematology analyser to measure platelet counts in conventional cGKI-null mutants (cGKF<sup>−/−</sup>), gene-targeted cGK<sub>α/β</sub> rescue mice (referred to as cGKI-smooth muscle [SM]) in which cGKI expression is specifically restored only in SM, platelet factor 4-Cre<sup>−/−</sup>; cGKF<sup>−/−</sup>-2<sup>−/−</sup> mice in which the cGKI protein was specifically deleted in the megakaryocyte/platelet lineage and cGKI-deficient bone marrow–chimeras. Thrombocytosis was detected in cGKF<sup>−/−</sup>-2<sup>−/−</sup> and in cGKI-SM. In contrast, neither platelet factor 4-Cre<sup>−/−</sup>; cGKF<sup>−/−</sup>-2<sup>−/−</sup> nor cGKI-deficient bone marrow–chimeras displayed a thrombocytosis phenotype, indicating that the high platelet count in cGKF<sup>−/−</sup>-2<sup>−/−</sup> and cGKI-SM mutants is attributable to loss of an extrinsic signal rather than reflecting an intrinsic defect in megakaryopoiesis. Cytometric analyses further showed that stimulation of bone marrow–derived wild-type megakaryocytes in vitro using serum preparations obtained from cGKI-SM mutants strongly accelerated megakaryopoiesis, suggesting that the high platelet count develops in response to serum factors. Indeed, using ELISA assay, we found elevated levels of interleukin-6, a known stimulator of thrombopoiesis, in cGKI-SM mutant serum, whereas interleukin-6 levels were unaltered in platelet factor 4-Cre<sup>−/−</sup>; cGKF<sup>−/−</sup>-2<sup>−/−</sup> mice and cGKI-deficient bone marrow–chimeras. Accordingly, antibody-mediated blockade of interleukin-6 normalized platelet counts in cGKI-SM mice.

Conclusions—Abnormal cGMP/cGKI signaling in nonhematopoietic cells affects thrombopoiesis via elevated interleukin-6 production and results in thrombocytosis in vivo. Dysfunction of cGMP/cGKI signaling in nonhematopoietic cells contributes to a high platelet count, which is potentially associated with thrombosis. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: cGMP ■ cGMP-dependent protein kinase type I ■ interleukin-6 ■ thrombopoiesis
proteins and granules. Together, these changes give rise to the platelet production machinery of mature MKs that release thrombocytes into the bone marrow (BM)–sinusoid vessels via long cytoplasmic processes, termed proplatelets (PPs). Fundamental functions of cAMP/cGMP signaling in mature platelets have been recognized for many years; however, the role of cAMP or cGMP in the maturation of MKs and the production of platelets in vivo are much less known. Recent in vitro findings indicate that platelet release from fetal liver–derived MKs is enhanced by high cGMP, but blocked by high cAMP levels in response to PGI₂, or agonists that directly activate adenylyl cyclase. As in many other cell types, the rate of cGMP production through soluble guanylyl cyclase activity and its degradation via cGMP-hydrolyzing phosphodiesterases (PDEs) determine the amplitude, duration, and spatiotemporal distribution of an intraplatelet cGMP signal. Of the 3 known platelet PDE isoforms, 2 isoforms, PDE-2 and PDE-3, may maintain an intensive cross-talk between nitric oxide (NO)/soluble guanylyl cyclase/cGMP and PGI₂/adenylyl cyclase/cAMP because these PDEs limit, with different affinities for their substrates and with different catalytic rates, the levels of both cyclic nucleotides, producing either inactive 5′-AMP or 5′-GMP. Most effects of high intraplatelet cGMP are directly mediated by cGMP-dependent protein kinase type I (cGKI), which is presumably the major effector of NO/cGMP in many cells. Interestingly, mature platelets express only the cGKILβ isozyme, whereas in numerous other cell types, such as hippocampal neurons and vascular or visceral smooth muscle (SM) cells, 2 cGKI isoforms, cGKIα and cGKILβ, act downstream of NO and cGMP. In platelets, NO/cGMP signaling via cGKI efficiently suppresses most agonist-induced and Ca²⁺-dependent activation/aggregation steps. However, the effect of cGKI on megakaryopoiesis and platelet biogenesis in vivo is not completely known.

To study the role of cGMP/cGKI signaling in megakaryopoiesis and platelet production in vivo, we took advantage of several established or recently generated transgenic mouse lines. Interestingly, we detected high platelet counts in conventional cGKI-null mutants (cGKIL1/L1) and in adult cGKI-SM mice with cGKI expression specifically restored in SM, but not in other cell types, whereas cGKI-null MKs developed normally under in vitro conditions. Moreover, neither platelet factor 4 (PF4)–Cre+/–; cGKIL1/L2 mice, in which cGKI was specifically ablated only in the MK/platelet lineage, nor cGKI-deficient BM chimera developed a similar thrombocytosis phenotype, indicating that the loss of cGKI in MKs and all radiosensitive hematopoietic cells does not contribute to thrombocytosis in cGKI-SM mutants. Our further investigations revealed that elevated IL-6 serum levels, which are known to enhance platelet production, are a plausible cause for thrombopoiesis in cGKI-SM mice because antibody-mediated inhibition of IL-6 normalized platelet counts in cGKI-SM but not in wild-type (WT) littermates. Taken together, our results suggest that the presence of cGKI in hematopoietic cells is not required for normal thrombopoiesis but the deficiency of cGKI in nonhematopoietic cells affects IL-6 turnover or production, resulting in a high platelet count in vivo. Our findings reveal a novel function of cGKI in thrombopoiesis and suggest that dysfunction of cGMP/cGKI signaling in nonhematopoietic cells leads to high platelet counts, which may lead to hemostatic problems.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Thrombocytosis in cGKI-Deficient Mice
We first confirmed that the cGKI protein was highly expressed in BM MKs and platelets (Figure 1A and 1B). To examine potential functions of cGKI in platelet biogenesis, we next measured total platelet counts in conventional cGKI-null mice. Platelet counts in 3-week-old cGKIL1/L1 mice were significantly higher as compared with WT littermates (Figure 1C). As reported previously, >50% of the cGKIL1/L1 null mutants die before 5 to 6 weeks of age; hence, to investigate the role of cGKI in thrombopoiesis in adult mice, and to bypass severe SM dysfunctions caused by the cGKI inactivation, we used a mouse line with restored expression of either the cGKIα or the cGKILβ isozyme specifically in SM, but not in other cell

Figure 1. Thrombocytosis in cGMP-dependent protein kinase type I (cGKI)–deficient mice. A, Representative immunohistochemical staining of cGKI (blue) in wild-type (WT; left) and cGKI-smooth muscle (SM; right) bone marrow. Arrows indicate stained megakaryocytes. Scale bars, 50 μm. B, Representative immunoblot for the cGKI protein in platelet lysates collected from WT and cGKI-SM mice. β-Actin was used to demonstrate equal loading of the gels. C, Platelet counts in peripheral blood from male WT littermates and cGKIL1/L1 mutants (n=7 WT littermates; n=4 cGKIL1/L1 mutants; SV129 background). D, Platelet counts (D) and mean platelet volume (E) in peripheral blood of female WT and cGKI-SM littermates (n=11 WT and n=10 cGKI-SM mice; SV129 background). All error bars represent SEM.
MK Polyploidization and MK Counts Are Increased in the BM of cGKI-SM Mice

Because the initial analyses of different cGKI mutants suggested that the thrombocytosis phenotype could be related to a primary defect in the developing MK/platelet lineage, we examined platelet life span and megakaryopoiesis in the peripheral blood and BM, respectively. Interestingly, the platelet life span in cGKI-SM mice was reduced rather than increased (Figure 2A), suggesting that the extent of platelet overproduction might be underestimated in vivo. It is possible that an enhanced platelet adhesion and aggregation in response to agonists in cGKI-deficient animals at least in part contributes to the faster clearance of platelets from the circulation observed here. The slightly shorter life span of cGKI-SM platelets (Figure 2A) together with the overproduction of platelets (Figure 1D) is likely to increase the proportion of new-born platelets in the whole platelet population in cGKI-SM mice, which explains the increase in platelet volume in these mutants (Figure 1E), given that newly formed platelets are bigger than mature platelets. Next, colony formation units–MK assays using mononuclear BM cells derived from cGKI-SM mice and WT littermates demonstrated that MK progenitor counts in both genotypes were comparable (Figure 2B). However, histological and flow cytometric analyses revealed that BM MK counts and the percentage of BM MKs with a DNA content ≥8 N were significantly elevated in cGKI-SM mice (Figure 2C–2E). These results indicate an enhanced megakaryopoiesis in cGKI-SM mice.

MK cGKI Is Not Required for Megakaryopoiesis and Thrombopoiesis In Vitro and Under Physiological Conditions In Vivo

To examine the role of cGKI for MK maturation in more detail, we used BM- or fetal liver–derived cells, and differentiated them into MKs in vitro. Given that the number of MK progenitors was not affected by cGKI ablation in the BM (Figure 2B), MK proliferation from BM-derived MK progenitors in vitro was similar for cGKI-null and WT cells (Figure 3A). In contrast to the higher polyploidization of cGKI-deficient MKs in vivo,
the polyploidization rate of cultivated BM-derived cGKI null and WT MKs was not different (Figure 3B). Next, we studied PP formation as an important functional property of MKs in fetal liver cells–derived MKs. Our data showed that the extent of PP formation was not affected by cGKI-deficiency (Figure 3C). To further investigate the function of cGKI for proplatelet fragmentation, which is the final stage of thrombopoiesis, we applied our previously described in vitro model, which enables us to monitor PP fragmentation events triggered by 10 μmol/L sphingosine 1-phosphate (S1P) using time-lapse video differential interference contrast microscopy. Our findings show that a similar extent of S1P-triggered PP fragmentation was observed in cGKI-deficient MKs and WT MKs (Figure 3D, Figure IA, and Movie I in the online-only Data Supplement), respectively, suggesting that cGKI is not involved in the regulation of S1P-induced PP fragmentation.

Together, the in vitro experiments did not support our initial hypothesis that cGKI plays an intrinsic role in thrombopoiesis. As a final test of whether cGKI signaling plays an intrinsic (autonomous) role in megakaryopoiesis in vivo, we generated gene-targeted mice with a MK- and platelet-specific cGKI ablation. Mice carrying floxed cGKI alleles (cGKIL2/L2) were crossed to Pf4-Cre transgenic mice, which express Cre recombinase under control of the MK-specific promoter Pf4. In conditional knockout animals (P4-Cre; cGKIL2/L2), the cGKI protein was specifically inactivated in platelets (Figure 3E), whereas other cardiovascular cell types that express cGKI were unaffected by the conditional ablation (Figure 3E). Although cGKI was efficiently inactivated in MK/platelets, our analysis showed that loss of cGKI in this lineage did not change platelet counts and mean platelet volume in the peripheral blood, confirming that the abnormal platelet counts in the peripheral blood of cGKIL1/L1 and cGKI-SM mice did not result from an intrinsic dysfunction of cGKI (Figure 3F and 3G). Notably, platelet counts in Pf4-Cre+/+; cGKIL2/L2 mice were slightly, but significantly higher than in WT littermates (Figure 1C and 1D, Figure 3F). This may result from different backgrounds of mouse lines because P4-Cre; cGKIL2/L2 and cGKI-SM mice were on an SV129/B6 background, whereas cGKIL1/L1 and cGKI-SM mice were on an SV129 background. Together with the in vitro findings, these data clearly rule out an intrinsic function of cGKI in platelet biogenesis.

**Thrombocytosis of cGKI-SM Mutant Mice Does Not Result From Defects in the Hematopoietic System**

Then we asked whether hematopoietic cells other than MKs contribute to thrombocytosis in cGKI-SM mice. To address this, we generated BM chimeras by transferring BM cells from...
WT littermates or conventional cGKI-null mice into lethally irradiated WT recipient mice. After 11 weeks, we observed a successful reconstitution of the recipient’s hematopoietic system with donor BM as indicated by anti-Ly9.1 and peripheral blood cytometric analyses (Figure 1B in the online-only Data Supplement). Interestingly, cGKI-null BM chimeras showed normal platelet counts and normal platelet size (Figure 4A and 4B). Therefore, cGKI-deficiency in radiosensitive hematopoietic cells does not cause the thrombocytosis phenotype of cGKI<sup>L1/L1</sup> and cGKI-SM mutant mice.

**Thrombocytosis in Gene-Targeted cGKI-SM Mutants Is Triggered by a Serum Factor**

It is known that factors produced and secreted by nonhematopoietic cells modulate MK and platelet biogenesis in vivo in a paracrine/endocrine manner.<sup>1,4</sup> To test this hypothesis, we analyzed BM-derived MKs cultured in serum-free medium supplemented with serum collected from either cGKI-SM or WT littermates. The cGKI-SM serum significantly increased the number of MKs in WT BM cell preparations in vitro (Figure 4C). Moreover, the stimulation of MKs using serum from cGKI-SM-SM mice significantly elevated the percentage of MKs with a DNA content of ≥4 N (Figure 4D), reminiscent of our observations in BM of cGKI-SM mice (Figure 2E). Thus, cGKI dysfunction in nonhematopoietic cells may cause a disturbed MK/platelet homeostasis via factors present in the blood serum of cGKI mutant mice.

**Enhanced IL-6 Serum Levels Contribute to Thrombocytosis in cGKI-SM Mice**

To clarify which serum factor enhances platelet biogenesis in cGKI-SM mice, we determined the serum levels of the key cytokines for megakaryocytopoiesis and platelet biogenesis in vivo, including Tpo and IL-6.<sup>24</sup> Serum Tpo levels in cGKI-SM mice were significantly reduced as compared with WT littermates (Figure 5A), whereas Tpo levels were unaltered in Pf4-Cre<sup>L6/L6</sup>; cGKI<sup>L2/L2</sup> mice or cGKI-deficient BM chimeras with their respective controls (Figure 5B and 5C). Given that free Tpo levels inversely correlate with platelet counts, lower serum Tpo levels are consistent with the higher number of platelets in cGKI-SM mice (Figure 1D).<sup>2</sup> As previously reported,<sup>25</sup> IL-6 serum levels were significantly elevated in cGKI-SM mice, whereas in cGKI-deficient BM chimeras and Pf4-Cre<sup>L6/L6</sup>; cGKI<sup>L2/L2</sup> mice, which did not develop a similar thrombocytosis phenotype (Figures 3F and 4A, respectively), IL-6 was detected at control levels (Figure 5D–5F). However, basal IL-6 levels were higher in BM chimeras than in non-transplanted animals most likely resulting from a chronic graft-versus-host immunoreactivity response after the allogeneic BM transplantations.<sup>26</sup> Notably, IL-6 concentrations did not differ between serum and plasma preparations from WT mice, excluding an effect of the platelet releasate on our above observations (data not shown).

It is well known that IL-6 enhances thrombopoiesis both in vitro and in vivo,<sup>27–29</sup> therefore, increased IL-6 serum levels could be a reason for the thrombocytosis phenotype in cGKI-SM mice. To prove this, we tested whether antagonizing IL-6 reduces the platelet counts of cGKI mutant mice. cGKI-SM mice were treated with an IL-6 neutralizing antibody, as previously reported, to block biological function of IL-6 in vivo.<sup>30,31</sup> Our targeted therapy indeed normalized the platelet counts in cGKI-SM mice, whereas the approach had no effects on platelet counts in WT littermates (Figure 5G). Together, these data suggest that elevated IL-6 serum levels enhance megakaryopoiesis and platelet production in cGKI-SM mice in vivo.

**Figure 4.** Serum factors contribute to enhanced thrombopoiesis. A, Platelet counts in peripheral blood from wild-type (WT) and cGMP-dependent protein kinase type I (cGKI)<sup>L1/L1</sup> bone marrow (BM) chimeras. Donor BM cells from cGKI<sup>L1/L1</sup> and WT female littermates (SV129 background) were transferred into WT female recipient mice (B6 background). The counts were performed 11 weeks after the transplantation (n=11 for WT BM chimeras and n=13 for cGKI<sup>L1/L1</sup> BM chimeras). B, Mean platelet volume in peripheral blood from WT and cGKI<sup>L1/L1</sup> BM chimeras. Donor BM cells from cGKI<sup>L1/L1</sup> and WT female littermates (SV129 background) were transferred into WT female recipient mice (B6 background). The measurements were performed 8 weeks after the transplantation (n=12 for WT BM chimeras and n=15 for cGKI<sup>L1/L1</sup> BM chimeras). C, Number of megakaryocytes (MKs) in cultured WT BM cells in response to blood serum obtained from cGKI WT or cGKI-ligand smooth muscle (SM) mice. Data represent the mean of 3 independent experiments performed in triplicate. D, The percentage of MK DNA ploidy in BM-derived MKs. Data represent the mean of 3 independent experiments performed in triplicate. All error bars represent SEM.
IL-6 Signaling Increases the Volume of MKs, But Has No Effect on MK Positioning, Motility, PP Formation, or PP Shedding In Vivo

Despite these well-known effects of IL-6 on megakaryopoiesis, the effect of IL-6 on dynamic MK behavior, for example, positioning, motility, PP formation, and shedding, has been insufficiently addressed in vivo. To test whether these dynamic MK functions are directly affected by high IL-6 levels, we performed multiphoton intravital microscopy. Using multiphoton intravital microscopy, we visualized MKs in calvarian BM from CD41-yellow fluorescent protein mice, in which MKs specifically express the yellow fluorescent protein under control of the endogenous CD41 gene promoter.

To analyze the interaction and localization of MKs in relation to the bloodstream, we labeled blood plasma by intravenous injection of 2 MDa tetramethylrhodamine isothiocyanate-dextran. In line with previous findings, we observed that the 3-dimensional volume of MKs from IL-6 treated mice was much larger compared with control mice (Figure 6A); however, IL-6 did not change the motility and positioning of MKs (Figure 6B and 6C, Movie II in the online-only Data Supplement), suggesting that IL-6 has no effect on MK migration.

We previously discovered that PPs polarize into BM sinusoid vessels in vivo, which is essential for efficient platelet production. Using multiphoton intravital microscopy, we detected that MKs from IL-6 treated and untreated mice protruded PPs into BM sinusoidal vessels (Figure 6D and 6E and Movie III in the online-only Data Supplement). Importantly, there was no difference between the treated and untreated control groups, indicating that IL-6 has no effect on polarization of PPs in vivo. Finally, the potential role of IL-6 for PP fragmentation and shedding was assessed. We observed PP shedding events in IL-6 treated mice as frequently as in untreated control mice (Figure 6F, Figure II, and Movie IV in the online-only Data Supplement), ruling out any effect of IL-6 on PP shedding. Taken together, neither migration nor polarization or fragmentation of PPs is disturbed by high IL-6 levels; however, IL-6 enlarges MK cell bodies in vivo, suggesting an effect on MK maturation.

Discussion

The cGMP-dependent protein kinase type I is an important downstream target of NO. The pleiotropic effects of NO in the cardiovascular system include modulating vessel tone, blood homeostasis, MK maturation, platelet formation, and platelet function. Pharmacological studies have shown that NO can induce apoptosis in MKs in vitro; however, these inhibitory effects of NO on MK growth were not dependent on cGMP but were mediated via proapoptotic factors, such as Bax, Bel-2, and peroxynitrite formation. Battinelli et al reported that NO increased production of platelet-like particles from a human megakaryocyte cell line and that cytokine-inducible nitric oxide synthase mice have reduced platelet counts, suggesting that NO is directly involved in thrombopoiesis in vivo.
cGKI specifically in the MK/platelet lineage and confirmed that MK cGKI does not intrinsically affect MK maturation or platelet production. In line with our present findings, Begonja et al\textsuperscript{16} have recently shown that the cGMP/cGKI pathway has no effect on MK development in vitro and does not affect MK proliferation or polyploidization. These authors further showed that a pharmacological stimulation of the cGMP pathway enhanced release of platelet-like particles from fetal liver cells–derived MKs,\textsuperscript{16} suggesting that the cGMP/cGKI pathway might play a role in platelet biogenesis. However, here we did not observe any effects of endogenous cGKI on platelet production under physiological conditions in different cGKI mutant mouse lines. Several reasons are likely to explain this discrepancy between our present observation and the previous report by Begonja et al\textsuperscript{16}: first, pharmacological overstimulation of the cGMP/cGKI pathway might indeed enhance platelet formation in vitro as suggested by Begonja et al\textsuperscript{16}; however, intrinsic MK cGMP/cGKI signaling should not be overstimulated under physiological conditions in vivo. Thus, we conclude from our in vivo data that the physiological activity of MK cGMP/cGKI pathway is not essential for thrombopoiesis. Nevertheless, overstimulation of cGMP/cGKI pathway might enhance platelet formation under pathophysiological conditions. Second, potential roles of cGKI in thrombopoiesis might be compensated by other pathways on cGKI inactivation in cGKI-deficient mice. Third, 8-pCPT-cGMP and BAY41-2272, which were used in the previous report, might activate other molecules downstream of cGMP besides cGKI pathway.

Although cGKI is not intrinsically required for thrombopoiesis in vivo, our results showed that the deficiency of cGKI in nonhematopoietic systems disturbed the homeostasis of platelet biogenesis in vivo. Elevated IL-6 serum levels were detected in cGKI-SM mice, but not in cGKI-deficient BM chimeras or Pf4-Cre\textsuperscript{65}; cGKI\textsuperscript{L2/L2} animals. In line with our observations, Lutz et al\textsuperscript{25} reported that the IL-6 serum levels were significantly increased in cGKI-SM mice probably by a mechanism involving cGMP/cGKI in liver, but not in skeletal muscles, spleen, or white adipose tissue.\textsuperscript{33} However, very recently it was shown that an inhibition of RhoA via cGMP/cGKI efficiently prevents the production of inflammatory factors, such as IL-6 in white adipocytes.\textsuperscript{40} The exact source and the cause of elevated IL-6 serum levels in cGKI mutant mice still remain to be identified in future studies.

Figure 6. The effect of interleukin-6 (IL-6) on thrombopoiesis in vivo. A, Volumes of megakaryocytes (MKs) in the indicated groups. The red lines indicate medians. Data were pooled from 4 to 5 mice per group. B, Instantaneous lateral (x-y) velocity of MKs. Data were pooled from 4 to 5 mice per group. C, Distance of MKs from bone marrow (BM) sinusoids. The red lines indicate medians. Data were pooled from 4 to 5 mice in each group. D, Intrasinusoidal PP formation in each group. MKs displaying intrasinusoidal PP formation (PPF) in vivo are presented as the percentage of all MKs carrying proplatelets (PPs; 43–60 MKs per group, 4–5 independent experiments per genotype). E, Representative in vivo images of yellow fluorescent protein (YFP)+ MKs (green) and vasculature (red) in mouse BM. Top row, CD41-YFP\textsuperscript{+} mice treated with PBS. Bottom row, CD41-YFP\textsuperscript{+} mice treated with IL-6. The arrowheads indicate intrasinusoidal proplatelet formation. Scale bars, 20 \textmu m. F, Percentage of PP fragmentation events observed by multiphoton intravital microscopy >1 hour in wild type×CD41-YFP\textsuperscript{+} treated with or without IL-6. Data are pooled from 3 to 4 independent experiments from each group (n= 20–40 per group). All error bars represent SEM.
IL-6 is an essential cytokine in the regulation of megakaryopoiesis and thrombopoiesis. Previous research showed that IL-6 can increase MK size and DNA ploidy in vitro. Liquid culture. Ishibashiet et al reported that IL-6 increased platelet counts and MK size in a dose-dependent fashion. Other studies confirmed that platelet production and MK numbers are elevated by increased IL-6 in mice. In humans, high IL-6 serum levels are 1 of the major causes for thrombocytosis. In line with these previous reports, an anti-IL-6 therapy normalized platelet counts in cGKI-SM mice (Figure 5G). This is a very important finding because age-cGKI-SM mice suffer from gastrointestinal bleedings, which might be related to severe hematological changes, including a high platelet count.

Despite these well-known effects of IL-6 on megakaryopoiesis, the role of IL-6 on in vivo functions of MKs, such as motility, positioning, and dynamic processes of platelet release, has not been investigated in detail. Using multiphoton intravital microscopy to visualize MKs within BM in vivo, we found that IL-6 increases the MK volume in vivo (Figure 6A). However, the migration of MKs and their localization in the BM were not altered by IL-6. In agreement with our findings, it has been demonstrated that fibroblast growth factor 4 and stromal cell–derived factor 1α rather than megakaryocyte-active cytokines, including Tpo, Il-6, or interleukin-11, play a predominant role for the localization of MK progenitors to the vascular niche, eventually promoting thrombopoiesis. Our previous studies reveal that the chemotactic S1P gradient controls polarization of PPs into BM sinusoid vessels via the S1P/S1pr1 signaling pathway. Once MKs extend PPs into the blood stream, shear stress and S1P promote PP shedding into the circulation. Here, we report that IL-6 affected neither polarization of PPs into BM sinusoid vessels nor PP fragmentation or shedding. Thus, elevated IL-6 serum levels may increase platelet production and megakaryopoiesis by boosting MK proliferation and polyploidization, but not by controlling the processes that guide platelet formation.

Taken together, our findings indicate that cGKI is not intrinsically required for homeostasis of platelet biogenesis, but the deficiency of cGKI in nonhematopoietic systems leads to a non-autonomous thrombocytosis phenotype, which is most likely driven by chronically elevated IL-6 serum levels. These results provide new insights into the cGMP/cGKI pathway and its role for the pathophysiology of thrombocytosis and its causes.

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We show here that in addition to its intrinsic effects on platelet function, cGMP-dependent protein kinase type I extrinsically controls platelet numbers in peripheral blood. We demonstrate that loss of cGMP-dependent protein kinase type I in nonhematopoietic system leads to thrombocytosis attributable to the elevated serum interleukin-6, a known stimulator of thrombopoiesis. Our findings reveal a novel function of cGMP-dependent protein kinase type I in thrombopoiesis and provide a new understanding of the pathophysiology of thrombocytosis, which has the potential to be associated with both thrombosis and paradoxical bleeding.
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Supplementary figure I.
(a) DIC microscopic analysis of proplatelet shedding in the presence of 10 μM S1P or its vehicle. MKs were kept in serum-free medium on a heated micro-incubator to keep the temperature at 37°C and monitored. A DIC microscope system equipped with a 40 × oil objective lens with NA = 0.7 was used to monitor PP shedding in real-time. Arrowhead, platelets released from PP stems; the upper row indicates WT MKs treated with vehicle, the middle row indicates WT MKs treated with S1P, and the lower row indicates cGKI^L^L^1^ MKs treated with S1P. All scale bars are 20 μm; Time in minutes.
(b) Flow cytometry analysis of chimaerism in BM chimaeras. The upper panel shows the gating strategy to analyse the percentage of CD229.1-positive cells in total CD4-positive T cells. In the lower panel, the first histogram shows the representative result from mice with C57Bl/6J background; the second one shows the sample prepared by mixing the blood from mice with C57Bl/6J background and the blood from mice with SV129 background; the last two show the representative CD229.1 chimaerism in CD4-positive T cells in WT C57Bl/6J mice reconstituted with WT (SV129 background) BM or cGKI^L^L^1^ (SV129 background) BM, respectively.

Supplementary figure II. Role for IL-6 in PP shedding in vivo visualized by MP-IVM.
Images were captured through a 20 × water immersion objective lens with NA = 0.95 using a BioTech TriM Scope system. Arrowheads and arrows indicate the tips of proplatelets. Green, MKs and PP; Red, Sinusoids; All scale bars, 20 μm; Time in minutes. The dashed line highlights the sinusoids.

Supplementary movie I: exogenous S1P induces proplatelet fragmentation in WT and cGKI^L^L^1^ MKs. 10 μM S1P induced proplatelet
fragmentation in both WT and cGKI<sub>1/1</sub> MKs within 1 h. MKs were kept on a heated micro-incubator to keep the temperature at 37°C in serum-free medium and monitored using a DIC microscope system equipped with a 40 × oil objective lens with NA = 0.7. The arrowhead indicates platelet-like particles separated from the MK stems. The arrow indicates the proplatelet stem between platelet-like particles. Time in minutes. Scale bar, 20 µm. 25 frames/seconds.

**Supplementary movie II: Positioning of MKs in calvarial bone marrow.** Intravital two-photon imaging of YFP+ MKs (green) and vasculature (red) in calvarial bone marrow of naïve (non-transplanted) CD41-YFP<sup>ki/+</sup> mice treated with or without IL-6. 3D movie was reconstructed from a stack of 10 sections at axial (z) spacing of 3 µm with a rotation from −30° to +30°. The arrowheads indicate intrasinusoidal YFP+ proplatelets. Scale bar, 20 µm.

**Supplementary movie III: MKs extend proplatelets preferentially into BM sinusoids.** Intravital two-photon imaging of YFP+ MKs (green) and vasculature (red) in calvarial bone marrow of CD41-YFP<sup>ki/+</sup> mice treated with or without IL-6. The 3D movie was reconstructed from a stack of 10 sections at axial (z) spacing of 3 µm with a rotation from −30° to +30°. The arrowheads indicate intrasinusoidal YFP+ proplatelets. Scale bar, 20 µm.

**Supplementary movie IV: Proplatelet shedding in vivo.** Proplatelet shedding in sinusoidal vessels in CD41-YFP<sup>ki/+</sup> mice treated with or without IL-6. YFP+ MKs (green) and vasculatures (red) in calvarial bone marrow were visualized by MP-IVM. The movie represents sequential images of the compressed stacks along the z axis (5 sections, z step of 5 µm). The arrows indicate intravascular proplatelets released from MKs. The dashed line highlights the sinusoids. Time in minutes. Scale bar, 20 µm. 25 frames/seconds.
Supplementary Figure I.

(a) Images showing the effect of WT + S1P and cGK1/L1 + S1P on cell morphology over time, with panels for 0min, 10min, 20min, 40min, 50min, and 60min.

(b) Flow cytometry plots for different cell populations:
- SV129 background: Lymphocyte population 99.75%, CD4+ population 0.25%
- C57Bl/6J background: Lymphocyte population 99.64%, CD4+ population 0.36%
- SV129 + C57Bl/6J blood: Lymphocyte population 63.19%, CD4+ population 36.81%
- WT BM chimaeras: Lymphocyte population 99.29%, CD4+ population 0.71%
- cGK1/L1 BM chimaeras: Lymphocyte population 99.24%, CD4+ population 0.76%
Supplementary Figure II.

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IL-6
Mice. C57BL6/J (B6) mice were purchased from Charles River Laboratories. Conventional cGKI null mutants (genotype: cGKI<sup>L1/L1</sup>) and cGKIα/β SM rescue animals (cGKI-SM; genotype: SM22α<sup>+/+cGKI</sup>; cGKI<sup>L1/L1</sup> and SM22α<sup>+/+cGKI</sup>); cGKI<sup>L1/L1</sup>, SV129 background) were generated as reported before<sup>1</sup>. For experiments gene-targeted cGKI<sup>L1/L1</sup> mice were compared to their age and littermate controls (WT; genotype: cGKI<sup>+/L1</sup> and/or cGKI<sup>+/+</sup>). To generate cGKI mutants with recombined cGKI-null alleles (L1) only in the MK/platelet lineage, animals with floxed cGKI alleles (genotype: cGKI<sup>L2/L2</sup>, SV129 background) were crossed to transgenic Pf4-Cre mice<sup>2</sup>, which carried one floxed cGKI allele (genotype: Pf4-Cre<sup>tg/+</sup>; cGKI<sup>+/L2</sup>, SV129/B6 background). The Pf4 promoter restricts the expression of Cre recombinase specifically to MKs and platelets, thus, this breeding strategy allowed us to analyse conditional cGKI knockouts (genotype: Pf4-Cre<sup>tg/+</sup>; cGKI<sup>L2/L2</sup>; SV129/B6 background) for their thrombocytosis phenotype in comparison to littermate control mice (genotype: Pf4-Cre<sup>+</sup>; cGKI<sup>L2/L2</sup>; SV129/B6 background). CD41-YFP<sup>ki/+</sup> reporter mice and BM chimaeras were generated as described before<sup>3,4</sup>. Briefly, we transferred WT or conventional cGKI null mutants (both on a SV129 background) BM cells into lethally irradiated C57Bl/6J mice. We used an anti-Ly9.1 antibody (BD) to determine the percentage of chimaerism, since the Ly9.1 antigen is present only on SV129-derived hematopoietic stem cells<sup>5</sup>. Chimaerism was greater
than 99% and confirmed by cytometric analysis showing that more than 99% of the blood cells were derived from donors. Age- and gender-matched mice were used in all studies. All animals were maintained and bred in the animal facilities of the Institut für Pharmakologie und Toxikologie, Technische Universität München, and Medizinische Klinik und Poliklinik I, Klinikum der Universität, Ludwig-Maximilians-Universität, Munich, Germany and had free access to water and standard chow. The experimental procedures were approved by the local government’s committee on animal care and welfare in Oberbayern.

**Blood cell analysis and serum TPO and IL-6 measurements.** Blood cell parameters were measured by using an automated cell counter (Sysmex Deutschland GmbH). Serum TPO and IL-6 measurements were performed using the Quantikine murine TPO or IL-6 Immunoassay Kits (R&D Systems) according to the manufacturers’ recommendations. To neutralize IL-6 in vivo, we treated mice with anti-mouse IL-6 antibody (50 µg/mouse i.p.) (Biolegend) or corresponding IgG control (Biolegend) every two days for three injections total. Platelet counts were assessed 1 week prior to anti-mouse IL-6 antibody or IgG administration and 2 days after the last administration.

**Immunohistochemical staining of BM.** Immunodetection was performed on paraffin-embedded serial 10 µm femur sections as previously published,
following an additional decalcification step\textsuperscript{6}. Biotinylated secondary antibodies (1:200 dilution; Vector Laboratories) in combination with the avidin-biotin method (Vector Laboratories) were used to identify primary antibody/antigen complexes by IHC. Visualization of the immune complexes was carried out by Vector Blue substrate (Vector Laboratories) or diaminobenzidine (brown staining) (Sigma-Aldrich). Primary antibodies used were specific for cGKI (1:50 dilution)\textsuperscript{1} and CD41 (1:1000 dilution, Santa-Cruz).

**Megakaryocyte cultivation.** Mouse fetal liver cells were isolated from E12.5-14.5 embryos and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% charcoal treated fetal bovine serum (FBS) (PAN, Aidenbach, Germany) in the presence of 100 ng/ml TPO (ImmunoTools, Friesoythe, Germany) in a humidified 5% CO\textsubscript{2}/95% air incubator at 37°C. We isolated BM cells from femurs of 3 weeks old cGKI\textsuperscript{L1/L1} mice and their corresponding WT littermates, or 12 weeks old WT mice and collected lineage-negative BM cells using a Lineage depletion kit (Miltenyi) according to the manufacturers’ recommendations. Then we cultivated lineage-negative BM cells in Iscove’s Modified Dulbecco’s Medium (IMEM) supplemented with 2% FBS (STEMCELL technolgoies) in the presence of 20 ng/ml stem cell factor (Sigma) for 2 days. The cells were cultivated for an additional 3 days under the same conditions in the presence of 20 ng/ml stem cell factor (Sigma) and 50 ng/ml TPO. This method was
applied to the experiments in Figure 3A-B. We distinguished and scored PPs according to the criteria mentioned previously\(^7\). To cultivate MKs with serum obtained from WT or cGKI-SM mice, BM lineage-negative cells were first isolated, as mentioned above. BM lineage-negative cells were then cultivated in StemSpan® SFEM medium (STEMCELL technologies) with 50 ng/ml TPO overnight. On the next day, the BM cell cultures were washed with PBS three times, and then an equal number of BM nucleated cells was cultivated in StemSpan® SFEM medium (STEMCELL technologies) supplemented with 5% (Figure 4C) or 10% (Figure 4D) mouse serum obtained from WT or cGKI-SM serum for 3 days (Figure 4C) or 1 day (Figure 4D).

**Megakaryocyte colony-forming unit (CFU-MK) assay.** We seeded 10,000 BM mononuclear cells isolated from murine femurs into Megacult-C medium (Stem Cell Technologies) and scored CFU-MKs on day 5 according to the manufacturer’s instructions.

**Flow cytometric analysis of platelet turn-over and MK polyploidization.** Platelet life span assays were described previously\(^4\). Briefly, mice were injected with N-hydroxysuccinimide-biotin (30 mg/kg) (Pierce, Bonn, Germany) via the lateral tail vein. After *in vivo* biotinylation, 20 µl blood samples were collected at different time points by retro-orbital bleed using capillaries and heparin-tubes and diluted 1:25 with PBS. Biotinylated platelets were detected
by simultaneous binding of phycoerythrin(PE)/Cy7-conjugated streptavidin (eBioscience, CA, USA) and FITC-conjugated antibodies to CD41. The percentage of biotinylated platelets on the first day was determined and used to normalize the percentage of biotinylated platelets on subsequent days to calculate the percentage of platelets remaining. The platelet half-lives and life spans of platelets were determined by linear regression of the data. For polyploidization analysis, BM-derived primary MKs were stained with FITC-anti-CD41 antibody (BD), and fixed in 1% PFA. The samples were incubated in DNA staining solution supplemented with 2 mM MgCl₂ (Sigma), 0.05% Saponin (Sigma), 0.01 mg/ml PI (Invitrogen) and 10 U/ml RNAse A (Qiagen) in PBS. After overnight incubation, the samples were measured by a FACSscalibur flow cytometer. As for enumeration of MK, the BM-derived cells were first stained with APC-anti-CD41 (BD) and Alexa-488-anti-CD42c (Emfret Analytics). 100 µl Accucount beads (Invitrogen) were added to each sample with the same volume. The number of events positive for both CD41 and CD42c was calculated after accumulation of 3, 000 Accucount beads.

**Western Blot analysis.** Aorta and platelets were isolated from experimental mice sacrificed by CO₂ inhalation. The vessels or platelets were lysed in 1 x RIPA lysis buffer (Cell signaling). 30µg of the lysates were resolved by SDS-PAGE and immunblotted using anti-cGKI (dilution 1:200) \(^1\), β-actin
(dilution 1:10,000) (Abcam) or anti-Gapdh (Sigma) and corresponding secondary antibodies (used in 1:2,500 dilutions).

**Two-photon intravital imaging of the BM.** We prepared mouse calvarial windows to image bone marrow according to the protocol described previously\(^4\). Briefly, mice were initially anaesthetized in an incubation chamber containing 5.0% Vol. Isofluran (Forene®, Abbott GmbH) and oxygen (3%). Prolonged Anaesthesia was initialized by intraperitoneal injection of a solution containing midazolam (5 mg/kg body weight, Ratiopharm) and medetomidine (0.5 mg/kg body weight, Pfizer). The hair on the skullcap was removed with hair removal lotion (MAXIM). A PE-10 catheter was placed into the tail vein for injection of liquids. After skin incision the front parietal skull was exposed and a plastic ring was inserted in the incision. The mouse head was immobilized on a custom-made stereotactic holder. We used a BioTech TriM Scope system based on a Ti: Sa laser (MaiTai, Spectra Physics) and with TriM Scope Scanhead (LaVision BioTec, Bielefeld, Germany) to capture images through a 20× water immersion objective lens with NA = 0.95 (Olympus). YFP was detected using a laser at a wavelength of 920 nm through a 525/50 nm filter. Vasculature was visualized by immediate administration of 2 MDa Tetramethylrhodamine-isothiocyanato-dextran (TRITC-dextran, Invitrogen). We used a laser at a wavelength 800 nm or 920 nm to excite TRITC-dextran and detected the emission signal through a 560-615 nm filter. Images were
acquired with Imspector software (LaVisionBioTec, Bielefeld, Germany). For three-dimensional acquisition, the stacks were first acquired at 920 nm at a vertical spacing of 3 µm to cover an axial depth of 30-100 µm (for YFP), and then the same stacks were immediately acquired at 800 nm wavelength (for TRITC). For analysis of PPF fragmentation efficiency, all the four-dimensional acquisitions were performed at 920 nm wavelength by capturing 3D image stacks at axial (z) distance of 5 µm to form a volume of 300 × 300 × 25 µm at 60 second intervals for 60 min. To generate 4D movies, 3D image stacks were flattened along the z-axis as maximum intensity projections representing a “top” (x–y) view of the volume. Constructed movies were generated using different time point frames by Volocity (Improvision). Volocity was used to reconstruct 3D structures from the stacks in the green channel detected at wavelength 920 nm and the stacks in the red channel detected at wavelength 800 nm. The measurement of their volumes and their distance to the vasculature were performed in reconstructed 3D structures using Volocity. To measure the velocity of MKs, we used the Stackreg plugin of ImageJ (http://rsb.info.nih.gov/ij/) to correct motion-artifacts. The centroid positions (x-y) of MKs from a series of top-view (x-y) images were measured using ImageJ, and instantaneous lateral (x-y) velocity, a measure of cell motility determined by dividing the change in cell displacement between each frame by the time interval between frames, was quantified by the Chemotaxis and Migration Tool plugin (ibidi). IL-6 was injected i.p 1.7 µg/mouse (from the same
litters and at the same age) every 8 hours for 3 days before imaging\textsuperscript{8,9}. The control mice were administrated with saline before imaging.

**Live cell imaging.** Mature MKs were starved in serum-free medium in custom-made Petri-dishes coated with human fibrinogen (100 $\mu$g/ml, Sigma Aldrich) for 1 h before incubation with S1P or vehicle. Live cell imaging was performed as described previously\textsuperscript{4}. Briefly, MKs were kept on a heated micro-incubator to keep the temperature at 37$^\circ$C and monitored using a DIC microscope system (CarlZeiss), equipped with a 40× oil objective lens with NA = 0.7 (CarlZeiss).

**Statistics.** We used two-tailed type 2 t-test, Chi-square and Kolmogorov-Smirnov (KS) test to calculate P values. P values of less than 0.05 were considered as statistically significant. All error bars are represented as S.E.M..

**References.**


