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

Lin Zhang,* Robert Lukowski,* Florian Gaertner, Michael Lorenz, Kyle R. Legate, Katrin Domes, Elisabeth Angermeier, Franz Hofmann, Steffen Massberg

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>L1/L1</sup>), gene-targeted cGKIα/β rescue mice (referred to as cGKI-smooth muscle [SM]) in which cGKI expression is specifically restored only in SM, platelet factor 4-Cre<sup>W/Δ</sup>, cGKI<sup>L2/L2</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 cGKI<sup>L1/L1</sup> and in cGKI-SM. In contrast, neither platelet factor 4-Cre<sup>W/Δ</sup>, cGKI<sup>L2/L2</sup> nor cGKI-deficient bone marrow—chimeras displayed a thrombocytosis phenotype, indicating that the high platelet count in cGKI<sup>L1/L1</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>W/Δ</sup>, cGKI<sup>L2/L2</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;33:1820-1828.)

Key Words: cGMP-dependent protein kinase type I ■ interleukin-6 ■ thrombopoiesis

A high platelet count has the potential to be associated with thrombosis and paradoxical bleeding. Clinically significant thrombocytosis is characterized by a high platelet count resulting from a multitude of pathogeneses, including infections, chronic inflammation, malignancies or iron deficiency and myeloproliferative disorders.1 Megakaryopoiesis and thrombopoiesis are tightly regulated by serum factors, such as thrombopoietin2 which acts in concert with several cytokines (eg, interleukin-3, interleukin-6, and interleukin-11) to maintain differentiation, proliferation, maturation of megakaryocytes, and the release of platelets.3,4 Megakaryopoiesis involves megakaryocyte lineage commitment from pluripotent hematopoietic stem and progenitor cells, proliferation of megakaryocyte progenitors, complex maturation steps of megakaryocytes and, finally, the release of platelets into the blood.3,4 Hematopoietic stem and progenitor cells give rise to a common megakaryocyte/erythroid progenitor.5 Megakaryocyte/erythroid progenitors further differentiate into highly proliferative potential colony forming unit–megakaryocytes, burst-forming unit–megakaryocytes, and colony forming unit–megakaryocytes with an increasing lineage specification.6–10 Colony formation units–megakaryocytes eventually differentiate into megakaryoblasts, which are characterized by a reduced proliferation capacity and >2 N DNA ploidy.10 DNA polyploidization results from nuclear endomitosis and is accompanied by extensive morphological and cytoskeletal changes involving an increase in cell size, the development of a complex membranous network known as the demarcation...
membrane system, and the synthesis of platelet-specific proteins and granules. Together, these changes give rise to the platelet production machinery of mature megakaryocytes that release thrombocytes into the bone marrow (BM)–sinusoid vessels via long cytoplasmic processes, termed proplatelets.

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 megakaryocytes and the production of platelets in vivo is much less known. Recent in vitro studies indicate that platelet release from fetal liver cells–derived megakaryocytes is enhanced by high cGMP, but blocked by high cAMP levels in response to prostaglandin I₂ or agonists that directly activate adenyl 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 high platelet 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 cGKI-dependent protein kinase type I (cGKI), which is presumably the major effector of NO/cGMP in many cells.

Interestingly, mature platelets express only the cGKIβ isozyme, whereas in numerous other cell types, such as hippocampal neurons and vascular or visceral smooth muscle (SM) cells, 2 cGKI isoforms, cGKIα and cGKIβ, act downstream of NO and cGMP. In platelets, NO/cGMP signaling via cGKIβ efficiently opposes most agonist-induced and Ca²⁺-dependent 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 (cGKI<sup>L1/L1</sup>) and in adult cGKI-SM mice with cGKI expression specifically restored in SM, but not in other cell types, whereas cGKI-null megakaryocytes developed normally under in vitro conditions. Moreover, neither platelet factor 4 (PF4) or cGKI<sup>L1/L1</sup> mice, in which cGKI was specifically ablated only in the megakaryocyte/platelet lineage, nor cGKI-deficient BM chimeras developed a similar thrombocytosis phenotype, indicating that the loss of cGKI in megakaryocytes and all radiosensitive hematopoietic cells does not contribute to thrombocytosis in cGKI-SM mutants. Our further investigations revealed that cGKI-deficient BM chimeras and megakaryocytes have elevated interleukin-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 interleukin-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 interleukin-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 megakaryocytes 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 cGKI<sup>L1/L1</sup> mice were significantly higher as compared with WT littermates (Figure 1C). As reported previously, ~50% of the cGKI<sup>L1/L1</sup> 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

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**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. Arrow indicates 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 cGKI<sup>L1/L1</sup> mutants (n=7 WT littermates; n=4 cGKI<sup>L1/L1</sup> mutants; SV129 background). Platelet counts in 3-week-old cGKI<sup>L1/L1</sup> mice were significantly higher as compared with WT littermates. D. Mean platelet volume (EP) 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.
inactivation, we used a mouse line with restored expression of either the cGKIα or the cGKIβ isozyme specifically in SM, but not in other cell types.22 The so-called cGKIα/β SM rescue mice (cGKI-SM) showed a prolonged life expectancy and normal vascular and visceral SM functions, as compared with cGKF1/L1 null mutants.22 As expected, cGKI-SM mice displayed a similar thrombocytosis phenotype as cGKF1/L1 mutants (Figure 1D), excluding the potential contribution of age or SM defects to elevated platelet counts. In addition, platelet volumes were larger in cGKI-SM mice as compared with WT littermates (Figure 1E), indicating an essential function of cGMP/cGKI for megakaryopoiesis and thrombopoiesis in vivo.

**Megakaryocyte Polyploidization and Megakaryocyte 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 megakaryocyte/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.13 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–megakaryocyte assays using mononuclear BM cells derived from cGKI-SM mice and WT littermates demonstrated that megakaryocyte progenitor counts in both genotypes were comparable (Figure 2B). However, histological and flow cytometric analyses revealed that BM megakaryocyte counts and the percentage of BM megakaryocytes 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.

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

To examine the role of cGKI for megakaryocyte maturation in more detail, we used BM- or fetal liver–derived cells, and differentiated them into megakaryocytes in vitro. Given that the number of megakaryocyte progenitors was not affected by cGKI ablation in the BM (Figure 2B), megakaryocyte
proliferation from BM-derived megakaryocyte progenitors in vitro was similar for cGKI-null and WT cells (Figure 3A). In contrast to the higher polyploidization of cGKI-deficient megakaryocytes in vivo, the polyploidization rate of cultivated BM-derived cGKI null and WT megakaryocytes was not different (Figure 3B). Next, we studied proplatelet formation as an important functional property of megakaryocytes in fetal liver cells–derived megakaryocytes. Our data showed that the extent of proplatelet 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 proplatelet 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 proplatelet fragmentation was observed in cGKI-deficient megakaryocytes and WT megakaryocytes (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 proplatelet fragmentation.

Together, the in vitro experiments did not support our initial hypothesis that cGKI plays an intrinsic role in thrombocytosis. As a final test of whether cGKI signaling plays an intrinsic (autonomous) role in megakaryopoiesis in vivo, we generated gene-targeted mice with a megakaryocyte- and platelet-specific cGKI ablation. Mice carrying floxed cGKI alleles (cGKIL1/L1) were crossed to Pf4-Cre transgenic mice, which express Cre recombinase under control of the megakaryocyte-specific promoter Pf4. In conditional knockout animals (Pf4-Cre+/-; cGKIL1/L1), the cGKI protein was specifically ablated 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 megakaryocyte/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+/-; cGKIL1/L1 and Pf4-Cre+/-; cGKI-SM mice did not result from an intrinsic dysregulation of cGKI in this lineage. The number of PPs with or without fragmentation observed by differential interference contrast (DIC) microscopy in vitro during 1 hour in the indicated groups. Data are pooled from 8 to 10 independent experiments (n=24–110 per group). E, Immunoblot of the cGMP-dependent protein kinase type I (cGKI) protein in aorta or platelet lysates obtained from platelet factor 4 (Pf4)-Cre+/-; cGKI−/− or Pf4-Cre+/-; cGKI−/− mice. GAPDH was used to demonstrate equal loading of the gels. F, Platelet counts in peripheral blood from male Pf4-Cre+/-; cGKI−/− or Pf4-Cre+/-; cGKIL1/L1 mice. n=5 Pf4-Cre+/-; cGKI−/−; n=7 Pf4-Cre+/-; cGKIL1/L1; SV129/B6 background. G, Mean platelet volume of male Pf4-Cre+/-; cGKI−/− or Pf4-Cre+/-; cGKIL1/L1 mice. n=5 Pf4-Cre+/-; cGKI−/−; n=5 Pf4-Cre+/-; cGKIL1/L1; SV129/B6 background. All error bars represent SEM.

Figure 3. Normal megakaryocyte (MK) development in vitro. A, Number of mature MKs from cultured bone marrow (BM) lineage-negative cells in the presence of 50 ng/mL thrombopoietin (Tpo) in vitro. The experiments shown were performed 3x in triplicate using independent BM preparations. MKs were identified as CD41+CD42c+ using cytometry. B, The percentage of DNA ploidy in cultured BM lineage-negative cells in the presence of 50 ng/mL Tpo in vitro. Data represent the mean of 4 independent experiments performed in triplicate. C, The percentage of fetal liver–derived MKs displaying proplatelet (PP) formation (PPF). Data represent the mean of 4 independent experiments performed in triplicate. At least 500 MKs were counted per experiment. D, The number of PPs with or without fragmentation observed by differential interference contrast (DIC) microscopy in vitro during 1 hour in the indicated groups. Data are pooled from 8 to 10 independent experiments (n=24–110 per group). E, Immunoblot of the cGMP-dependent protein kinase type I (cGKI) protein in aorta or platelet lysates obtained from platelet factor 4 (Pf4)-Cre+/-; cGKI−/− or Pf4-Cre+/-; cGKI−/− mice. GAPDH was used to demonstrate equal loading of the gels. F, Platelet counts in peripheral blood from male Pf4-Cre+/-; cGKI−/− or Pf4-Cre+/-; cGKIL1/L1 mice. n=5 Pf4-Cre+/-; cGKI−/−; n=7 Pf4-Cre+/-; cGKIL1/L1; SV129/B6 background. G, Mean platelet volume of male Pf4-Cre+/-; cGKI−/− or Pf4-Cre+/-; cGKIL1/L1 mice. n=5 Pf4-Cre+/-; cGKI−/−; n=5 Pf4-Cre+/-; cGKIL1/L1; SV129/B6 background. All error bars represent SEM.
Thrombocytosis of cGKI-SM Mutant Mice Does Not Result From Defects in the Hematopoietic System

Then we asked whether hematopoietic cells other than megakaryocytes 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 IB 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 megakaryocyte and platelet biogenesis in vivo in a paracrine/endocrine manner. To test this hypothesis, we analyzed BM-derived megakaryocytes 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 megakaryocytes in WT BM cell preparations in vitro (Figure 4C). Moreover, the stimulation of megakaryocytes using serum from cGKI-SM mice significantly elevated the percentage of megakaryocytes with a DNA content of 4N (Figure 4D), reminiscent of our observation in BM of cGKI-SM mice (Figure 2E). Thus, cGKI dysfunction in nonhematopoietic cells may cause a disturbed megakaryocyte/platelet homeostasis via factors present in the blood serum of cGKI mutant mice.

Enhanced Interleukin-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 megakaryocytogenesis and platelet biogenesis in vivo, including thrombopoietin and interleukin-6. Serum thrombopoietin levels in cGKI-SM mice were significantly reduced as compared with WT littermates (Figure 5A), whereas thrombopoietin levels were unaltered in Pf4-Cre<sup>lox/lox;</sup> cGKI<sup>L1/L1</sup> mice or cGKI-deficient BM chimeras as compared with their respective controls (Figure 5B and 5C). Given that free thrombopoietin levels inversely correlate with platelet counts, lower serum thrombopoietin levels are consistent with the higher number of platelets in cGKI-SM mice (Figure 1D). As previously reported, interleukin-6 serum levels were significantly elevated in cGKI-SM mice, whereas in cGKI-deficient BM chimeras and Pf4-Cre<sup>lox/lox;</sup> cGKI<sup>L1/L1</sup> mice, which did not develop a similar thrombocytosis phenotype (Figures 3F and 4A, respectively), interleukin-6 was detected at control levels (Figure 5D–5F). However, basal interleukin-6 levels were higher in BM chimeras than in nontransplanted animals most likely resulting from a chronic graft-versus-host immunoreactivity response after the allogeneic BM transplantations. Notably, interleukin-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 interleukin-6 enhances thrombopoiesis both in vitro and in vivo<sup>27–29</sup>; therefore, increased interleukin-6 serum levels could be a reason for the thrombocytosis phenotype in cGKI-SM mice. To prove this, we tested whether antagonizing interleukin-6 reduces the platelet counts of cGKI mutant mice. cGKI-SM mice were treated with an interleukin-6 neutralizing antibody, as previously reported, to block

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**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-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.
biological function of interleukin-6 in vivo.10,11 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 interleukin-6 serum levels enhance megakaryopoiesis and platelet production in cGKI-SM mice in vivo.

Interleukin-6 Signaling Increases the Volume of Megakaryocytes, But Has No Effect on Megakaryocyte Positioning, Motility, Proplatelet Formation, or Proplatelet Shedding In Vivo

Despite these well-known effects of interleukin-6 on megakaryopoiesis,27-29 the effect of interleukin-6 on dynamic megakaryocyte behavior, for example, positioning, motility, proplatelet formation, and shedding, has been insufficiently addressed in vivo. To test whether these dynamic megakaryocyte functions are directly affected by high interleukin-6 levels, we performed multiphoton intravital microscopy. Using multiphoton intravital microscopy, we visualized megakaryocytes in calvarian BM from CD41-yellow fluorescent protein mice,32 in which megakaryocytes specifically express the yellow fluorescent protein under control of the endogenous CD41 gene promoter. To analyze the interaction and localization of megakaryocytes in relation to the blood stream, we labeled blood plasma by intravenous injection of 2 MDa tetramethylrhodamine isothiocyanate-dextran.12 In line with previous findings,28 we observed that the 3-dimensional volume of megakaryocytes from interleukin-6 treated mice (1.7 μg/mouse every 8 hours for 3 days) was much larger as compared with control mice (Figure 6A); however, interleukin-6 did not change the motility and positioning of megakaryocytes (Figure 6B and 6C, Movie II in the online-only Data Supplement), suggesting that interleukin-6 has no effect on megakaryocyte migration. We previously discovered that proplatelets polarize into BM sinusoid vessels in vivo, which is essential for efficient platelet production.12 To examine the effect of interleukin-6 on the polarization of proplatelets, we next studied the distribution of proplatelets and their relationships to BM sinusoid vessels. Using multiphoton intravital microscopy, we detected that megakaryocytes from interleukin-6 treated and untreated mice protruded proplatelets 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 interleukin-6 has no effect on polarization of proplatelets in vivo. Finally, the potential role of interleukin-6 for proplatelet fragmentation and shedding was assessed. We observed proplatelet shedding events in interleukin-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 interleukin-6 on proplatelet shedding. Taken together, neither migration nor polarization or fragmentation of proplatelets is disturbed by high interleukin-6 levels; however, interleukin-6 enlarges megakaryocyte cell bodies in vivo, suggesting an effect on megakaryocyte 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, megakaryocyte maturation, platelet formation, and platelet function. Pharmacological studies have shown that NO can induce apoptosis in megakaryocytes in vitro; however, these inhibitory effects of NO on megakaryocyte growth were not dependent on cGMP but were mediated via proapoptotic factors, such as Bax, Bcl-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 deficient mice have reduced platelet counts, suggesting that NO is directly involved in thrombopoiesis in vivo. Using a conditional approach, we targeted cGKI specifically in the megakaryocyte/platelet lineage and confirmed that megakaryocyte cGKI does not intrinsically affect megakaryocyte maturation or platelet production. In line with our present findings, Begonja et al have recently shown that the cGMP/cGK1 pathway has no effect on megakaryocyte development in vitro as suggested by Begonja et al; however, intrinsic megakaryocyte cGMP/cGK1 signaling should not be stimulated under physiological conditions in vivo. Thus, we conclude from our in vivo data that the physiological activity of megakaryocyte cGMP/cGK1 pathway is not essential for thrombopoiesis. Nevertheless, overstimulation of cGMP/cGK1 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 cGK1-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 interleukin-6 serum levels were detected in cGKI-SM mice, but not in cGKI-deficient BM chimeras or Pf4-Cre+/−; cGKI−/− animals. In line with our observations, Lutz et al23 reported that the interleukin-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.21 However, very recently it was shown that an inhibition of RhoA via cGMP/cGKI efficiently prevents the production of inflammatory factors, such as interleukin-6 in white adipocytes.40 The exact source and the cause of elevated interleukin-6 serum levels in cGKI mutant mice still remain to be identified in future studies.

Interleukin-6 is an essential cytokine in the regulation of megakaryopoiesis and thrombopoiesis. Previous research showed that interleukin-6 can increase megakaryocyte size and DNA ploidy in in vitro liquid culture.41 Ishibashiet al reported that interleukin-6 increased platelet counts and megakaryocyte size in a dose-dependent fashion.28 Other studies confirmed that platelet production and megakaryocyte numbers are elevated by increased interleukin-6 in mice.20 In humans, high interleukin-6 serum levels are 1 of the major causes for thrombopoiesis.1 In line with these previous reports,27–29 an anti–interleukin-6 therapy normalized platelet counts in cGKI-SM mice (Figure 5G). This is a very important finding because with age cGKI-SM mice suffer from gastrointestinal bleedings, which might be related to severe hematological changes, including a high platelet count.48

Despite these well-known effects of interleukin-6 on megakaryopoiesis, the role of interleukin-6 on in vivo functions of megakaryocytes, such as motility, positioning, and dynamic processes of platelet release, has not been investigated in detail. Using multiphoton intravital microscopy to visualize megakaryocytes within BM in vivo, we found that interleukin-6 increases the megakaryocyte volume in vivo (Figure 6A).28 However, the migration of megakaryocytes and their localization in the BM were not altered by interleukin-6. In agreement with our findings, it has been demonstrated that fibroblast growth factor 4 and stromal cell–derived factor-1 rather than megakaryocyte-active cytokines, including thrombopoietin interleukin-6, or interleukin-11, play a predominant role for the localization of megakaryocyte progenitors to the vascular niche, eventually promoting thrombopoiesis.49 Our previous studies reveal that the chemotactic SIP gradient controls polarization of proplatelets into BM sinusoid vessels via the SIP/SIPr1 signaling pathway.12 Once megakaryocytes extend proplatelets into the blood stream, shear stress and SIP promote proplatelet shedding into the circulation.12,44 Here, we report that interleukin-6 affected neither polarization of proplatelets into BM sinusoid vessels nor proplatelet fragmentation or shedding. Thus, elevated interleukin-6 serum levels may increase platelet production and megakaryopoiesis by boosting megakaryocyte proliferation and polyplidization, 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 nonautonomous thrombocytosis phenotype, which is most likely driven by chronically elevated interleukin-6 serum levels. These results provide new insights into the cGMP/cGKI pathway and its role for the pathophysiology of thrombocytosis and its causes.

Acknowledgments
We thank Anne Suhr for excellent technical assistance. L. Zhang, R. Lukowski, K. Legare, F. Hofmann, and S. Massberg designed the research and conception of the study; analyzed data; and wrote and reviewed the article. L. Zhang, R. Lukowski, F. Gaertner, K. Domes, and E. Angermeyer performed experiments.

Sources of Funding
This study was supported by the Deutsche Forschungsgemeinschaft with grants to R. Lukowski, F. Hofmann, and S. Massberg (Sonderforschungsbereich 914 to S. Massberg and Forschergruppe 923 to F. Hofmann and S. Massberg) and by the European Commission (FP7 project Prestige to S. Massberg).

Disclosures
None.

References
Significance

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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Arterioscler Thromb Vasc Biol. 2013;33:1820-1828; originally published online June 20, 2013; doi: 10.1161/ATVBAHA.113.301507
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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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\textsuperscript{L1/L1} 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\textsuperscript{L1/L1} (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\textsuperscript{L1/L1} MKs. 10 µM S1P induced proplatelet
fragmentation in both WT and cGK1L/L 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-YFPki/+ 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-YFPki/+ 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-YFPki/+ 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

WT + vehicle

0min 10min 20min 40min 50min 60min

WT + S1P

0min 10min 20min 40min 50min 60min

cGKIl/L1 + S1P

0min 10min 20min 40min 50min 60min

b

SV129 background

Lymphocyte population

CD4+ population

SV129 background

99.75%

0.25%

SV129 background

C57BL/6J background

SV129 + C57BI/6J blood

WT BM chimaeras

cGKIl/L1 BM chimaeras

0.36%

99.64%

36.81%

63.19%

0.71%

99.29%

36.81%

63.19%

0.71%

99.29%

0.76%

99.24%
Materials and methods

Mice. C57BL6/J (B6) mice were purchased from Charles River Laboratories. Conventional cGKI null mutants (genotype: cGKI\(^{L1/L1}\)) and cGKI\(\alpha/\beta\) SM rescue animals (cGKI-SM; genotype: SM22\(\alpha^+/cGKI\), cGKI\(^{L1/L1}\) and SM22\(\alpha^+/cGKI\), cGKI\(^{L1/L1}\), SV129 background) were generated as reported before\(^1\). For experiments gene-targeted cGKI\(^{L1/L1}\) mice were compared to their age and littermate controls (WT; genotype: cGKI\(^{+/L1}\) and/or cGKI\(^{+/+}\)). To generate cGKI mutants with recombined cGKI-null alleles (L1) only in the MK/platelet lineage, animals with floxed cGKI alleles (genotype: cGKI\(^{L2/L2}\), SV129 background) were crossed to transgenic Pf4-Cre mice\(^2\), which carried one floxed cGKI allele (genotype: Pf4-Cre\(^{tg/+}\); cGKI\(^{+/L2}\), 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\(^{tg/+}\); cGKI\(^{L2/L2}\); SV129/B6 background) for their thrombocytosis phenotype in comparison to littermate control mice (genotype: Pf4-Cre\(^{+/+}\); cGKI\(^{L2/L2}\);SV129/B6 background). CD41-YFP\(^{ki/+}\) reporter mice and BM chimaeras were generated as described before\(^3,4\). 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\(^5\). 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. 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) 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 \(\mu\)l 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 FACScalibur 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 × 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 previously⁴. 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 920nm 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 µg/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°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.**


