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Abstract

To explore the functional significance of cGMP-dependent protein kinase type I (cGKI) in the regulation of erythrocyte survival, gene-targeted mice lacking cGKI were compared with their control littermates. By the age of 10 weeks, cGKI-deficient mice exhibited pronounced anemia and splenomegaly. Compared with control mice, the cGKI mutants had significantly lower red blood cell count, packed cell volume, and hemoglobin concentration. Anemia was associated with a higher reticulocyte number and an increase of plasma erythropoietin concentration. The spleens of cGKI mutant mice were massively enlarged and contained a higher fraction of Ter119(+) erythroid cells, whereas the relative proportion of leukocyte subpopulations was not changed. The Ter119(+) cGKI-deficient splenocytes showed a marked increase in annexin V binding, pointing to phosphatidylserine (PS) exposure at the outer membrane leaflet, a hallmark of suicidal erythrocyte death or eryptosis. Compared with control erythrocytes, cGKI-deficient erythrocytes exhibited in vitro a higher cytosolic Ca(2+) concentration, a known trigger of eryptosis, and showed increased PS exposure, which was paralleled by a faster clearance in vivo. Together, these results identify a role of cGKI as mediator of erythrocyte survival and extend the emerging concept that cGMP/cGKI signaling has an antiapoptotic/prosurvival function in a number of cell types in vivo.
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Anemia and splenomegaly in cGKI-deficient mice

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Running title: cGKI and erythrocyte survival

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Abstract

To explore the functional significance of cGMP-dependent protein kinase type I (cGKI) in the regulation of erythrocyte survival, gene targeted mice lacking functional cGKI were compared to their control littermates. By the age of 10 weeks, cGKI-deficient mice exhibited pronounced anemia and splenomegaly. Compared to control mice, the cGKI mutants had a significantly lower red blood cell count, packed cell volume and hemoglobin concentration. Anemia was associated with a higher reticulocyte number and an increase of plasma erythropoietin concentration. The spleens of cGKI mutant mice were massively enlarged and contained a higher fraction of Ter119^+ erythroid cells, whereas the relative proportion of leukocyte subpopulations was not changed. The Ter119^+ cGKI-deficient splenocytes showed a marked increase in annexin V-binding pointing to breakdown of phosphatidylserine asymmetry, a hallmark of suicidal erythrocyte death or eryptosis. Indeed, cGKI-deficient erythrocytes showed in vitro a higher sensitivity to eryptosis than control cells. According to Fluo-3 fluorescence, the cytosolic Ca^{2+} concentration, a known trigger of eryptosis, was increased in erythrocytes from cGKI knockout mice. Transfer experiments with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled erythrocytes showed that the in vivo clearance of cGKI-deficient erythrocytes was faster than that of control cells. Together, these results identify a novel role of cGKI as mediator of erythrocyte survival and extend the emerging concept that cGMP/cGKI signaling has an anti-apoptotic/pro-survival function in a number of cell types in vivo.

Key words: apoptosis, cell growth, erythrocyte, nitric oxide, PKG
Introduction

Nitric oxide (NO) has previously been shown to be a powerful regulator of cell survival [Dimmeler et al., 2002; Hoffmann et al., 2001; Liu and Stamler 1999; Traister et al., 2004]. Depending on the source or concentration of NO and on the influence of additional regulators, NO may stimulate or inhibit apoptosis [Brune 2003]. NO exerts its effects in part through S-nitrosylation of target proteins. However, the effect of NO donors on Ca\(^{2+}\)-induced phosphatidylserine exposure, a hallmark of apoptosis, could be mimicked by cGMP analogues [Nagai-Kusuhara et al., 2007], suggesting the involvement of soluble guanylyl cyclase, cGMP and the cGMP-dependent protein kinase type I (cGKI), a well-known signalling cascade downstream of NO [Fleming et al., 1999; Friebe and Koesling 2003; Schlossmann et al., 2003].

Erythroid cells possess a functional NO/cGMP pathway [Chen and Mehta 1998; Ikuta et al., 2001; Kleinbongard et al., 2006], which thus may be involved in the regulation of eryptosis, the suicidal death of erythrocytes [Lang et al., 2005]. Eryptosis may follow activation of Ca\(^{2+}\)-permeable cation channels [Lang et al., 2003a] and subsequent Ca\(^{2+}\) entry leading to activation of Ca\(^{2+}\)-sensitive K\(^+\) channels, exit of KCl with osmotically obliged water and thus cell shrinkage [Lang et al., 2003b]. In addition, the Ca\(^{2+}\) entry triggers the Ca\(^{2+}\)-sensitive scrambling of the cell membrane [Bratosin et al., 2001; Woon et al., 1999] with subsequent exposure of phosphatidylserine at the erythrocyte surface [de Jong et al., 2002; Lang et al., 2003a; Matarrese et al., 2005]. Cell membrane scrambling may further be triggered by ceramide [Lang et al., 2004] and activation of protein kinase C [Klarl et al., 2006]. Phosphatidylserine-exposing erythrocytes bind to phosphatidylserine receptors [Fadok et al., 2000] and are thus recognised, engulfed, and degraded by macrophages [Boas et al., 1998]. Accordingly, similar to erythrocyte senescence [Barvitenko et al., 2005; Bosman et al., 2005; Schwarzer et al., 2005] and neocytolysis [Rice and Alfrey 2005], eryptosis leads to clearance of affected erythrocytes from circulating blood.

The present study has been performed to explore the role of cGKI in the survival of circulating erythrocytes. To this end, experiments have been performed to compare gene targeted mice lacking functional cGKI with their control littermates.

Results

The arterial acid base status as well as the most important plasma electrolytes (Na, K, Ca) did not differ between both mouse lines (data not shown).

The analysis of peripheral blood showed significant erythrocyte abnormalities in conventional cGKI knockout (cGKI ko) mice as compared to litter-matched control (ctr) mice. The red blood cell (RBC) counts, hematocrit (HCT), and hemoglobin (HGB) concentration were significantly smaller in 10-week-old cGKI ko mice than in ctr mice (Fig. 1A). The difference was larger for erythrocyte number (≈52%) than for packed cell volume (≈33%) and hemoglobin (≈31%). Accordingly, the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin content (MCH) of an erythrocyte were higher in cGKI ko mice than in ctr mice, whereas the mean corpuscular hemoglobin concentration (MCHC) was not altered. Thus, lack of cGKI
leads to marked anemia. In theory, the anemia could have resulted from decreased erythrocyte formation, which should be reflected by a decreased number of reticulocytes. However, the reticulocyte number was higher in cGKI ko than in ctr mice (Fig. 1B). Consistent with reticulocytosis, the red blood cell distribution width (RDW) was increased in cGKI ko mice (Fig. 1A). Thus, the anemia could not be explained by decreased formation of erythrocytes. The increased reticulocyte number in cGKI ko mice could have resulted from enhanced stimulation of erythropoiesis by erythropoietin. As illustrated in Fig. 1C, the plasma erythropoietin concentration was indeed about twofold higher in cGKI ko than in ctr mice. The increased reticulocyte and erythropoietin levels could be indicative of hemolytic anemia in cGKI ko mice. To explore this possibility, the plasma concentration of haptoglobin was determined. The haptoglobin concentration in anemic cGKI ko mice was similar in both genotypes (in mg/ml, cGKI ko 0.11 ± 0.02, n=8; ctr 0.12 ± 0.03, n=11) excluding a hemolytic nature of the observed anemia. Moreover, iron deficiency due to chronic bleeding or impaired iron absorption is unlikely to account for the anemia, since the plasma transferrin levels were again similar in both genotypes (in mg/ml, cGKI ko 1.17 ± 0.05, n=8; ctr 1.37 ± 0.08, n=7). Moreover, the erythrocytes of cGKI ko mice were not microcytic (Fig.1A).

The anemia of cGKI-deficient mice was associated with severe splenomegaly. Based on the organ/body weight ratio, 10-week-old cGKI ko mice had ~3-fold larger spleens than control mice, whereas the size of the heart and kidneys was normal (Fig. 2A,B). Analysis of individual animals revealed that the splenomegaly was evident in most cGKI ko mice aged 8 to 10 weeks (Fig. 2C). It is important to note that conventional cGKI ko mice have a severe smooth muscle (SM) dysfunction, which causes premature death of ~50% of the mutant animals by 6 weeks of age. To test whether the anemia and splenomegaly of cGKI ko mice was linked to their SM dysfunction and poor health status, another cGKI-deficient mouse model, the so-called cGKI SM rescue mouse [Weber et al., 2007], was also analysed. In cGKI SM rescue mice, the expression of cGKI has been restored selectively in SM cells but not in other cell types resulting in the rescue of SM dysfunction and a dramatic extension of life span to more than one year [Weber et al., 2007]. Thus, cGKI SM rescue mice are a useful genetic model to study the role of cGKI in non-SM cells of relatively “healthy” mice. Similar to “older” conventional cGKI ko mice, adult (8- to 40-week old) cGKI SM rescue mice were anemic (RBC in 10⁶/μl, ctr 9.9 ± 0.2, n=22; SM rescue 7.7 ± 0.3, n=17; P<0.001) and showed massive splenomegaly at all ages analysed (Fig. 2C). The fact that the anemia and splenomegaly were also present in the relatively “healthy” cGKI SM rescue mouse model indicated that these defects were not secondary due to the poor health condition of conventional cGKI ko mice and that they are not the cause of their premature death.

Further experiments were performed to elucidate the characteristics of the cells that apparently accumulated in the enlarged spleens of the cGKI-deficient mice. Freshly prepared splenocytes were stained with an antibody against Ter119, a marker of late-stage murine erythroid cells and mature erythrocytes and with annexin V to examine the externalization of phosphatidylserine (PS) indicative of suicidal cell death. Flow-cytometric analysis showed that cGKI mutant mice had, proportionally, ~96% more Ter119+ splenocytes than ctr mice (Fig. 2D, Table 1). When corrected for the increased spleen size in cGKI mutants, this reflects a ~6-fold increase in spleen erythroid cell mass. Annexin V-binding indicated that Ter119+ splenocytes of cGKI-
deficient animals contained, proportionally, twice as much PS-exposing cells than ctr mice (PS-exposing erythroid cells / erythroid cells, SM rescue ≈20%, ctr ≈9%), whereas nonerythroid cells showed no significant difference in the level of annexin V-labeled cells between genotypes (Table 1). Immunophenotyping for marker proteins indicated that the relative proportion of splenic CD41+ megakaryocytes [Tiedt et al., 2007], CD4+ T cells, CD8+ T cells, and B220+ B cells as well as the proliferative activity of the splenocytes in the absence and presence of lipopolysaccharide (LPS) were similar in ctr and cGKI-deficient animals (Table 1). Moreover, the analysis of splenocytes for intracellular and secreted cytokines did not reveal altered levels of pro-inflammatory (IL-2, IL-17, IFN-γ, TNF) or anti-inflammatory (IL-4, IL-10) mediators in the spleens of cGKI mutants compared to controls (data not shown). Thus, the increased spleen size of the cGKI-deficient mice was not due to an increase in nonerythroid cells including lymphocytes, but was caused, at least in large part, by a massively increased number of eryptotic erythrocytes.

The accumulation of erythrocytes in the spleens of cGKI-deficient animals could be linked to their higher sensitivity to eryptosis. Therefore, it was explored whether cGKI signaling plays a role in eryptosis. Western blot analysis showed that cGKI protein is present in both, erythroid cells from the bone marrow as well as in peripheral erythrocytes (Fig. 3). To exclude a potential contamination of the erythroid cells with platelets containing a high level of cGKI [Feil et al., 2003; Hofmann et al., 2006], the same membranes were stained for the platelet marker, thrombospondin-1 (TSP-1). While TSP-1 was readily detected in platelet-rich plasma, it was not present in the erythroid cell preparations used for Western analysis, thus, excluding the contamination with platelets (Fig. 3). Following a 48 hours incubation in Ringer solution at 37°C, the percentage of PS-exposing erythrocytes was determined by flow cytometry. Erythrocytes from cGKI ko mice showed a significantly higher percentage of PS-exposing erythrocytes than ctr mice (Fig. 4A). Externalization of PS is stimulated by an increased cytosolic Ca^{2+} concentration [Lang et al., 2003a]. Ca^{2+} imaging with Fluo-3 showed that the cytosolic Ca^{2+} concentration was indeed significantly higher in cGKI ko erythrocytes than in ctr cells (Fig. 4B). These data suggested that cGKI inhibits erythrocyte death in vitro by reducing the cytosolic Ca^{2+} concentration, which in turn limits the exposure of PS at the cell surface. To investigate whether cGKI deficiency also affected the mechanical properties of the erythrocytes, cGKI ko and ctr erythrocytes were exposed to shear stress and the resulting cell elongation as a measure of erythrocyte flexibility was determined using laser-defractometry. The cGKI ko erythrocytes were slightly but significantly more flexible than ctr cells. For instance, the % elongation at a shear stress of 3.1 Pa in a high viscosity solution (24.4 mPa*s) were 24 ± 0.4 in cGKI ko and 19 ± 0.3 in ctr erythrocytes (P<0.05), whereas the % elongation at a shear stress of 2.6 Pa in a low viscosity solution (10.4 mPa*s) were 17 ± 0.2 in cGKI ko and 14 ± 0.4 in ctr erythrocytes (P<0.05). Since the deformability of cGKI-deficient erythrocytes was not impaired and in fact slightly improved, their mechanical properties could not account for their accumulation in the spleens of cGKI mutant mice.

The previous experiments suggested that cGKI deficiency promotes the death of erythrocytes, which might accumulate in the spleens of the mutant animals, thus leading to splenomegaly. To determine their in vivo survival, erythrocytes were isolated from control or cGKI ko donor mice, labeled with carboxyfluorescein...
diacetate succinimidyl ester (CFSE), and subsequently injected into the tail vein of ctr or SM rescue recipient mice. The clearance of CFSE-labeled erythrocytes from the circulation, an indirect indicator of erythrocyte death, was monitored over 24 days (Fig. 5). In line with their higher susceptibility to eryptosis in vitro, the cGKI ko erythrocytes disappeared more rapidly in ctr mice than ctr erythrocytes in ctr mice in vivo. As expected, ctr erythrocytes injected into cGKI mutant mice with splenomegaly were cleared very rapidly, most likely due to their retention in the enlarged spleens of the recipient mice (Fig. 5). Together, these data suggested that the anemia of cGKI-deficient mice is not only due to an erythrocyte-autonomous component, i.e. increased eryptosis, but is potentiated by the development of an enlarged spleen retaining more erythrocytes than a normal spleen. Along those lines, anemia but not splenomegaly was detectable in 3- to 4-week-old cGKI ko mice (Fig. 6), whereas 10-week-old knockout mice were both anemic and had enlarged spleens (Figs. 1A and 2A-C). Thus, it appears that the anemia due to eryptosis precedes the splenomegaly in cGKI-deficient mice.

Discussion

The present study shows that cGKI-deficient mice develop anemia and splenomegaly. The anemia is most likely not due to impaired formation but rather due to decreased survival of erythrocytes. The increased levels of plasma erythropoietin and reticulocytes indicate that erythropoiesis is indeed activated in cGKI-deficient mice, but eventually cannot compensate for the loss of erythrocytes resulting in anemia. Ter119+ erythroid cells with an increased level of PS-exposure accumulate in the spleens of the mutant animals explaining the enlarged spleens and supporting a role of cGKI signaling in erythrocyte survival. Indeed, cGKI-deficient erythrocytes show a decreased survival both in vitro and in vivo. Mechanistically, the decreased survival of cGKI-deficient erythrocytes might result from an increased intracellular Ca$^{2+}$ concentration, which triggers the exposure of PS at the erythrocyte surface followed by engulfment by splenic macrophages.

The cytosolic Ca$^{2+}$ concentration is higher in circulating erythrocytes from cGKI-deficient mice as compared to ctr mice. It is well known that the NO/cGMP/cGKI pathway inhibits agonist-induced increases in cytosolic Ca$^{2+}$ in vascular smooth muscle cells [Feil et al., 2002; Pfeifer et al., 1998]. Many studies have suggested that this pathway decreases the cytosolic Ca$^{2+}$ concentration also in a variety of other cell types and that the Ca$^{2+}$ lowering effect might, at least in part, be due to inhibition of Ca$^{2+}$ entry through Ca$^{2+}$-permeable channels [Abdallah et al., 2005; Ay et al., 2006; Dervaux et al., 2006; Gomes et al., 2006; Huang et al., 2006; Kwan et al., 2006; Ma and Wang 2006]. Moreover, cGMP stimulates the Ca$^{2+}$ ATPase [Dedkova and Blatter 2002; Ogurusu et al., 1990; Rashatwar et al., 1987; Uneyama et al., 1998; Vrolix et al., 1988], participating in the regulation of erythrocyte Ca$^{2+}$ concentration [Davis et al., 1982; Xu and Roufogalis 1988]. In line with their increased cytosolic Ca$^{2+}$ concentration, which is a well-known trigger of phospholipid scrambling [Lang et al., 2003a], cGKI-deficient erythrocytes show increased surface exposure of PS, a hallmark of eryptosis [Bratosin et al., 2001; Woon et al., 1999]. The exposure of PS at the erythrocyte surface mediates the binding to PS receptors of macrophages [Fadok et al., 2000] followed by engulfment [Boas et al., 1998] and clearance from circulating blood [Kempe et al., 2006]. Indeed, cGKI-deficient erythrocytes are more rapidly cleared from the circulation, thus leading to the development of anemia.
The mechanical properties of cGKI-deficient erythrocytes were not affected adversely and, therefore, could not contribute to their accumulation in the spleen.

These results disclose a novel role of endogenous cGKI as an anti-apoptotic component in the regulation of erythrocyte survival. Cyclic GMP and cGKI have been previously shown to counteract apoptosis of nucleated cells [Nagai-Kusuhara et al., 2007]. The mechanism suppressing eryptosis via cGKI and lowering of the cytosolic Ca\(^{2+}\) concentration may be similarly effective in the regulation of apoptotic death of nucleated cells [Perretti and Solito 2004]. Needless to say that the cell death machinery in nucleated cells is more complex than in erythrocytes and, thus, the mechanisms shown effective in erythrocytes may be complemented by additional mechanisms in nucleated cells. Although indirect effects, e.g. resulting from altered erythropoiesis or defects in nonerythroid cells of the cGKI mutants, cannot be completely ruled out, several findings suggest a direct effect of cGKI on the survival of mature erythrocytes. First, the eryptosis phenotype is observed in isolated mature erythrocytes indicative of an erythrocyte-autonomous defect. Secondly, the anemia and splenomegaly is similarly present in “sick” conventional cGKI knockout mice and in relatively “healthy” cGKI smooth muscle rescue mice. Third, the anemia appears to precede the splenomegaly excluding the possibility that the development of a large spleen and the associated increased clearance of erythrocytes is the primary cause of the anemia. Thus, we propose that the primary defect leading to anemia in cGKI-deficient mice is a higher susceptibility of mature erythrocytes to eryptosis, which results in accumulation of the apoptotic cells in the spleen and, thereby, in its enlargement and further retention of erythrocytes. In this way, a vicious cycle is generated in which anemia and splenomegaly potentiate each other to reach the level observed in the adult cGKI mouse mutants.

In conclusion, the present study demonstrates that lack of functional cGKI in erythrocytes leads to an increased cytosolic Ca\(^{2+}\) concentration with subsequent breakdown of PS asymmetry, accelerated clearance of circulating erythrocytes, and anemia despite counterregulation by erythropoietin release. These observations identify a novel function of cGKI signaling in the regulation of erythrocyte survival and support the emerging concept that pathways via NO, cGMP, and cGKI stimulate cell growth and survival in a number of cell types in vivo [Feil et al., 2005a; Fiedler et al., 2006; Wolfsgruber et al., 2003; Yamahara et al., 2003]. The positive effect of cGKI on erythrocyte survival also suggests novel therapeutic options to treat anemia with cGMP-elevating drugs.
Materials and Methods

**Experimental animals and cells**

Experiments were performed with 3- to 10-week-old conventional cGKI knockout mice [Wegener et al., 2002] carrying the L- null allele (cGKI ko mice, genotype: cGKI\(^{L-}\)) and with 8- to 45-week-old cGKI smooth muscle rescue mice [Weber et al., 2007], in which the expression of the cGKI\(\alpha\) or cGKI\(\beta\) isozyme was selectively restored in smooth muscle but not in other cell types of cGKI\(^{L-}\) mice (SM-\(\alpha\) or SM-\(\beta\) rescue mice, genotype: cGKI\(^{L-}\);SM-\(\alpha\) or cGKI\(^{L-}\);SM-\(\beta\)). As controls, litter- and gender-matched mice with the following genotypes were used: for cGKI ko mice, wild-type (cGKI\(^{+/+}\)) mice and heterozygous cGKI (cGKI\(^{+/-}\)) mutants, and for SM rescue mice, mice expressing endogenous cGKI as well as the respective SM-\(\alpha\) or SM-\(\beta\) transgene (SM-\(\alpha\) or SM-\(\beta\) control mice, genotype: cGKI\(^{+/+}\);SM-\(\alpha\) or cGKI\(^{+/+}\);SM-\(\beta\)). The results did not significantly differ between male and female mice of the same genotype, between SM-\(\alpha\) and SM-\(\beta\) rescue mice, between cGKI\(^{+/+}\) and cGKI\(^{+/-}\) control mice, and between SM-\(\alpha\) and SM-\(\beta\) control mice. Therefore, data were pooled from both sexes, from SM-\(\alpha\) and SM-\(\beta\) rescue mice, from cGKI\(^{+/+}\) and cGKI\(^{+/-}\) control mice, and from SM-\(\alpha\) and SM-\(\beta\) control mice. All mice were on a 129/Sv genetic background. All experimental procedures were conducted according to the local government’s committee on animal care and welfare.

Blood was retrieved either by puncture of the retroorbital venous plexus or, in case of sacrificed mice, by puncture of the heart, and collected in heparin- or EDTA-coated tubes. Bone marrow was obtained by rinsing femur and tibia with PBS. Cells from the bone marrow and spleen were separated with Netwells (Corning) prior to further analysis.

**Blood and plasma parameters**

Erythrocyte number, packed cell volume, mean corpuscular volume and blood hemoglobin concentration were determined using an electronic hematology particle counter (type MDM 905 from Medical Diagnostics Marx; Butzbach, Germany) equipped with a photometric unit for hemoglobin determination. The gating has been adjusted for the application on mouse erythrocytes. These measurements were confirmed by the measurement of hematocrit by centrifugation at 15000g for 3 minutes, measuring the hemoglobin concentration photometrically at 546nm after adding of 20 \(\mu\)l blood to 3 ml of a hemoglobin transformation solution (Dr. Lange AG, Hegnau, Switzerland) and by counting the red cells manually after 1:400 dilution with Heyem’s solution (Fluka, Buchs, Switzerland) in a Neubauer chamber. Using the hematocrit, hemoglobin and red cell count obtained this way, the MCV, MCH and MCHC were calculated.

The relative distribution width (RDW) was determined from images of blood smears taken with a CCD camera (Axiocam, Zeiss, Deisenhofen, Germany) at a magnification of 400x (Axioskop, Zeiss). Of
1829 to 3444 individual erythrocytes of each animal (n=3 to 4) the size was determined using an image analyzing system (MCID, Ontario, Canada). The obtained standard deviations and the means of the erythrocyte size were used to calculate the coefficient of variation as a measure for the RDW.

For determination of relative reticulocyte numbers, 5 µl of whole blood were added to 1 ml Retic-COUNT (Thiazole orange) reagent from Becton Dickinson. Samples were stained for 30 min at room temperature in the dark. Then, FACS analysis of stained samples was performed according to the manufacturer’s instructions. Forward scatter (FSC), side scatter (SSC), and thiazole orange-fluorescence intensity (in the fluorescence channel FL-1) of the blood cells were measured on a FACS-Calibur from Becton Dickinson. The numbers of Retic-COUNT positive reticulocytes were determined and relative reticulocyte numbers were expressed as the percentage of the total, gated erythrocyte populations. Gating of erythrocytes was achieved by analysis of FSC vs. SSC dot plots using the CellQuestTM software.

Arterial acid base status and the plasma concentration of sodium, potassium and calcium were determined with automatic blood gas analyzer (pHOx-plus, Nova Biomedical, Waltham, MA, USA).

The plasma concentration of erythropoietin, haptoglobin, and transferrin were determined using immunoassay kits according to the manufacturer’s instructions (erythropoietin: R&D systems, Wiesbaden-Nordenstadt, Germany; others: Kamiya Biomedical Company, Seattle, USA).

Analysis of spleens

Single cell suspensions of freshly isolated spleens were stained for surface markers and intracellular cytokines and analyzed by flow cytometry according to standard procedures. Antibodies to Ter119, CD41, CD3, CD4, CD8, B220, IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF, and appropriate isotype controls labeled with FITC or PE were obtained from BD, Germany. Annexin V-FITC staining was performed using an Apoptosis Detection Kit (BD, Germany). For intracellular cytokine staining, isolated splenocytes were stimulated with PMA (15 ng/ml) and ionomycin (1.5 µg/ml) (both Sigma, Germany) for 4 hours in the presence of Brefeldin A (Golgi Plug, BD, Germany). Cells were fixed in 2% formaldehyde, washed and permeabilized with 0.5% Saponin / 0.5% BSA and incubated with the indicated antibodies. After washing, 50,000 or 100,000 events were counted on a FACS Calibur (BD, Germany) and data were analyzed using CellQuest software (BD, Germany). For investigating the proliferative capacity of splenocytes, 1x10⁵ cells per well were cultured in medium or stimulated with LPS (1µg/ml). Cell proliferation was examined by measuring DNA synthesis using [³²H]thymidine incorporation (1.25 μCi/ml) for 12 hours before harvesting and counting using a MicroBeta device (Perkin Elmer, Germany).

Western blot analysis

Erythroid cells were isolated from bone marrow and peripheral blood by immunomagnetic selection using magnetically labelled Anti-Ter-119 MicroBeads and magnetic cell sorting (MACS, Miltenyi Biotec)
[Kina et al., 2000]. Proteins were separated on a SDS gel, transferred to a PVDF membrane and stained with a polyclonal rabbit antiserum to cGKI [Feil et al., 2005b] or with an antibody against thrombospondin-1 (TSP-1) (Lab Vision). As a positive control for both cGKI as well as TSP-1 expression, platelet-rich plasma was also loaded.

**Analysis of phosphatidylserine exposure and intracellular Ca\(^{2+}\) in peripheral erythrocytes**

Erythrocytes were washed two times in Ringer solution (in mM: 125 NaCl, 5 KCl, 1 MgSO\(_4\), 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl\(_2\), pH = 7.4). Then, erythrocytes at a final hemtocrit of 0.4% were incubated in Ringer solution at 37°C for 48 hours. After incubation, FACS analysis was performed essentially as described [Andree et al., 1990; Lang et al., 2003a]. For measurement of phosphatidylserine exposure, cells were washed in Ringer containing 5 mM Ca\(^{2+}\) and then stained with Annexin-V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 20 min, samples were measured by flow cytometry (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Annexin-V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For intracellular Ca\(^{2+}\) measurements, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in Ringer solution containing 5 mM CaCl\(_2\) and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 20 min under shaking and washed twice in Ringer solution containing 5 mM CaCl\(_2\). The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer solution. Then, Ca\(^{2+}\)-dependent fluorescence intensity was measured in FL-1. To test for Fluo-3 loading, ionomycin (1 µM) was applied at the end of the experiments. The ionomycin-evoked Fluo-3 fluorescence did not differ significantly between the genotypes (data not shown).

**Measurement of the half-life of fluorescence-labeled erythrocytes in vivo**

Erythrocytes were fluorescence-labeled by staining the cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Leiden, The Netherlands). The labeling solution was prepared by addition of a CFSE stock solution (10 mM in DMSO) to phosphate-buffered saline (PBS) to yield a final concentration of 5 µM. Erythrocytes were obtained from 200 µl blood of donor mice and incubated with labeling solution for 30 min at 37°C under light protection. The cells were pelleted at 400 g for 5 min, washed twice in PBS containing 1% FCS and pelleted at 400 g for 5 min. The pellet was then resuspended in Ringer solution (37°C) and 100 µl of the fluorescence-labeled erythrocytes were injected into the tail vein of the recipient mouse. After the respective time periods, blood was retrieved from the tail veins of the mice and CFSE-dependent fluorescence intensity of the erythrocytes was measured in FL-1 as described above. The percentage of CFSE-positive erythrocytes was calculated in % of the total erythrocyte number.
Measurement of erythrocyte flexibility

Freshly drawn blood (20 µl) was suspended in 2 ml phosphate buffered saline containing Dextran (MW 60000, Serva, Wallisellen, Switzerland) in amounts yielding a viscosity of 24.4 or 10.4 mPas*s (measured with a cone-plate viscosimeter, DVIII+ Rheometer, Brookfield Engineering Laboratories INC, Middlebrow, MA, USA). The osmolarity of these solution was adjusted to 310 mosm/L. The red cell / test solution suspension was transferred into a Laser defractometer (Myrenne, Röttgen, Germany) and the percent elongation of the erythrocytes was recorded at shear stresses between 0.31 and 61 s⁻¹ (24.4 mPa*s solution) or 0.13 and 26 s⁻¹ (10.4 mPa*s solution).

Statistics

Data are expressed as mean ± SEM and statistical analysis was made by two-tailed unpaired t test or by two way ANOVA followed by Bonferroni post hoc test if appropriate. Significance was determined at P<0.05.

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**Figure legends**

**Fig. 1.** Anemia in cGKI-deficient mice. Circulating blood of 10-week-old control (ctr, open bars) and cGKI ko (ko, black bars) mice was analysed. (A) Counts of red blood cells (RBC), hematocrit (HCT), hemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin content (MCH), mean corpuscular hemoglobin concentration (MCHC), and the red blood cell distribution width (RDW). The data shown were obtained from a litter-matched group of mice (n=3-4) and are representative for at least three experiments with independent groups of animals. (B) Representative histogram of Retic count fluorescence (left panel) and reticulocyte number (right panel, n=8). (C) Plasma erythropoietin concentration (n=4). *and ** indicates significant differences between genotypes with P<0.05 and P<0.01, respectively.

**Fig. 2.** Splenomegaly associated with increased erythroid cell mass in cGKI-deficient mice. (A) Spleens and (B) organ/body weight (bw) ratios of 10-week-old control (ctr, open bars) and cGKI ko (ko, black bars) mice (**, P<0.01). The data shown were obtained from a litter-matched group of mice (n=3-4) and are representative for at least three experiments with independent groups of animals. (C) Spleen/bw ratios of individual mice at various ages. The diagram includes conventional cGKI ko mice (black boxes) and their control littermates (open boxes) as well as cGKI SM rescue mice (black triangles) and their controls (open triangles). (D) Representative flow-cytometric quantification of Ter119⁺ spleen cells isolated from a 42-week-old cGKI SM rescue mouse and a litter-matched control mouse.

**Fig. 3.** Expression of cGKI in murine erythroid cells. Western blot analysis of cGKI expression in erythroid cells from a 26-week-old wild-type mouse. Protein extracts of platelet-rich plasma (5 µg) and of Ter119⁺ erythroid cells isolated from bone marrow (30 µg) or peripheral blood (30 µg) were stained with an antiserum raised against cGKI (lower panel) or thrombospondin-1 (TSP-1, upper panel).

**Fig. 4.** Increased eryptosis and intracellular Ca²⁺ level in cGKI-deficient erythrocytes. Peripheral erythrocytes were isolated from 4- to 6-week-old control (ctr) and cGKI ko (ko) mice and then incubated in Ringer solution for 48 hours before analysis. (A) Surface exposure of phosphatidylserine as determined by annexin V-binding and (B) measurement of intracellular Ca²⁺ by Fluo-3 fluorescence. The respective left panel shows a representative flow-cytometric histogram and the right panel shows the statistical analysis (n=12 for each measurement; ***, P<0.001).

**Fig. 5.** In vivo clearance of CFSE-labeled erythrocytes. Erythrocytes were isolated from control (ctr) or cGKI ko (ko) mice and then injected into ctr or cGKI SM rescue mice. The following combinations of
donor erythrocytes injected into recipient mice were performed: ctr into ctr (open boxes, n=4), ko into ctr (black triangles, n=4), and ctr into SM rescue (black diamonds, n=3). The percentage of CFSE-labeled cells is plotted against time after injection (**, P<0.01, ko into ctr vs. ctr into ctr at indicated time points). ANOVA showed a significant difference between experimental groups for both ko into ctr vs. ctr into ctr (P<0.001) as well as ctr into SM rescue vs. ctr into ctr (P<0.001).

**Fig. 6.** Anemia precedes splenomegaly in cGKI-deficient mice. The number of red blood cells (RBC, n=5) and the spleen/body weight (bw) ratio (n=10) was determined in 3- to 4-week-old control (open bars) and cGKI ko (black bars) mice (*, P<0.05).
Table 1. Cellular makeup of spleens from cGKI-deficient mice

<table>
<thead>
<tr>
<th></th>
<th>ctr</th>
<th>SM rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroid cells, % (Ter119⁺/total splenocytes)</td>
<td>28 ± 3.6</td>
<td>55 ± 6.5*</td>
</tr>
<tr>
<td>Apoptotic erythroid cells, % (Ter119⁺ &amp; annexin V⁺/total splenocytes)</td>
<td>2.5 ± 0.4</td>
<td>11 ± 0.7*</td>
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<tr>
<td>Apoptotic nonerythroid cells, % (Ter119⁻ &amp; annexin V⁺/total splenocytes)</td>
<td>27 ± 6.5</td>
<td>20 ± 3.5</td>
</tr>
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<td>CD41⁺ megakaryocytes, %</td>
<td>7.9 ± 2.0</td>
<td>10 ± 4.0</td>
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<tr>
<td>CD4⁺ T cells, %</td>
<td>31 ± 5.8</td>
<td>24 ± 4.9</td>
</tr>
<tr>
<td>CD8⁺ T cells, %</td>
<td>9.1 ± 1.4</td>
<td>6.1 ± 1.4</td>
</tr>
<tr>
<td>B220⁺ B cells, %</td>
<td>35 ± 4.1</td>
<td>30 ± 6.1</td>
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<tr>
<td>³H-thymidine incorporation</td>
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<tr>
<td>basal, cpm</td>
<td>448 ± 95</td>
<td>377 ± 109</td>
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<tr>
<td>LPS (1 µg/ml), cpm</td>
<td>6563 ± 1688</td>
<td>4781 ± 1854</td>
</tr>
</tbody>
</table>

All data are mean ± SEM (n = 3-5 mice). 34- to 45-week-old cGKI SM rescue mice and their control littermates were analysed. Statistical test results are reported as P value by t test. *, P<0.05 vs. ctr.