Blunted apoptosis of erythrocytes in mice deficient in the heterotrimeric G-protein subunit Go\(\alpha\)i2

Rosi Bissinger\(^1\), Elisabeth Lang\(^2\), Mehrdad Ghashghaeinia\(^3\), Yogesh Singh\(^4\), Christine Zelenak\(^5\), Birgit Fehrenbacher\(^6\), Sabina Honisch\(^7\), Hong Chen\(^8\), Hajar Fakhri\(^1\), Anja T. Umbach\(^1\), Guilai Liu\(^1\), Rexhep Rexhepaj\(^1,5\), Guoxing Liu\(^1\), Martin Schaller\(^6\), Andreas F. Mack\(^6\), Adrian Lupescu\(^1\), Lutz Birnbaumer\(^7\), Florian Lang\(^1\) & Syed M. Qadri\(^1,8,9\)

Putative functions of the heterotrimeric G-protein subunit Go\(\alpha\)i2-dependent signaling include ion channel regulation, cell differentiation, proliferation and apoptosis. Erythrocytes may, similar to apoptosis of nucleated cells, undergo eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine (PS) exposure. Eryptosis may be triggered by increased cytosolic Ca\(^{2+}\) activity and ceramide. In the present study, we show that Go\(\alpha\)i2 is expressed in both murine and human erythrocytes and further examined the survival of erythrocytes drawn from Go\(\alpha\)i2-deficient mice (Go\(\alpha\)i2\(^{-/-}\)) and corresponding wild-type mice (Go\(\alpha\)i2\(^{+/+}\)). Our data show that plasma erythropoietin levels, erythrocyte maturation markers, erythrocyte counts, hematocrit and hemoglobin concentration were similar in Go\(\alpha\)i2\(^{-/-}\) and Go\(\alpha\)i2\(^{+/+}\) mice but the mean corpuscular volume was significantly larger in Go\(\alpha\)i2\(^{-/-}\) mice. Spontaneous PS exposure of circulating Go\(\alpha\)i2\(^{-/-}\) erythrocytes was significantly lower than that of circulating Go\(\alpha\)i2\(^{+/+}\) erythrocytes. PS exposure was significantly lower in Go\(\alpha\)i2\(^{-/-}\) than in Go\(\alpha\)i2\(^{+/+}\) erythrocytes following ex vivo exposure to hyperosmotic shock, bacterial sphingomyelinase or C6 ceramide. Erythrocyte Go\(\alpha\)i2 deficiency further attenuated hyperosmotic shock-induced increase of cytosolic Ca\(^{2+}\) activity and cell shrinkage. Moreover, Go\(\alpha\)i2\(^{-/-}\) erythrocytes were more resistant to osmosensitive hemolysis as compared to Go\(\alpha\)i2\(^{+/+}\) erythrocytes. In conclusion, Go\(\alpha\)i2 deficiency in erythrocytes confers partial protection against suicidal cell death.

G protein-coupled receptors activate heterotrimeric G proteins via ligand binding, thereby modulating the activity of cellular effectors and consequently regulating a wide array of cell functions\(^{1,2}\). The putative function of the functional class of G protein Go\(i\) is defined by their ability to downregulate cAMP levels by inhibition of adenylyl cyclase\(^{2,3}\). The closely-related G\(i\) members Go\(i\)1, Go\(i\)2, and Go\(i\)3, sharing 85–95% of their amino acid sequence identity, are characterized by their sensitivity to pertussis toxin\(^{3,4}\). Go\(i\)2, the quantitatively predominant G\(i\) isoform, is a decisive regulator of leukocyte, endothelial and platelet functions\(^{5-7}\). Further putative roles of Go\(i\)2 signaling include ion channel regulation, cell differentiation, proliferation and apoptosis\(^{8-12}\). Effector kinases of G-protein signaling include phosphoinositide 3-kinases\(^{13}\), which are known to be involved in the regulation of apoptosis\(^{14}\). Go\(i\)2 further influences Ca\(^{2+}\) signaling in nucleated cells by the activation of TRPC4 channels which, in turn, increases Ca\(^{2+}\) influx\(^{15}\). In cardiomyocytes, Go\(i\)2 has been shown to modulate the activity of L-type

\(^{1}\)Institute of Cardiology, Vascular Medicine and Physiology, University of Tuebingen, Germany. \(^{2}\)Department of Gastroenterology, Hepatology and Infectious Diseases, University of Dusseldorf, Germany. \(^{3}\)Department of Internal Medicine, Charité Medical University, Berlin, Germany. \(^{4}\)Department of Dermatology, University of Tuebingen, Germany. \(^{5}\)Institute of Biochemistry and Molecular Biology, University of Bonn, Germany. \(^{6}\)Institute of Anatomy, University of Tuebingen, Germany. \(^{7}\)Neurobiology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, Durham, NC, USA. \(^{8}\)Institute of Biomedical Research (BIOMED), School of Medical Sciences, Catholic University of Argentina, Buenos Aires, Argentina. \(^{9}\)Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada. Correspondence and requests for materials should be addressed to F.L. (email: florian.lang@uni-tuebingen.de)
Voltage-dependent $\text{Ca}^{2+}$-channels\textsuperscript{11}. Furthermore, $\text{G}_{\alpha_i}^2$ is a powerful regulator of cytosolic $\text{Ca}^{2+}$ activity in islet beta cells\textsuperscript{12} and neutrophils\textsuperscript{16}, thus, regulating a variety of $\text{Ca}^{2+}$-dependent cell functions. Phenotypically, $\text{G}_{\alpha_i}^2$ knockout mice have been reported to display a predisposition towards a wide range of disorders including growth retardation, inflammatory bowel disease, carcinogenesis, cardiac arrhythmia and impaired haemostasis\textsuperscript{4,17,18}.

Similar to nucleated cells, erythrocytes may undergo suicidal death or eryptosis\textsuperscript{19,20}, which, similar to apoptosis, is triggered by osmotic shock and characterized by cell shrinkage and cell membrane scrambling\textsuperscript{20,21}. Eryptosis may be triggered by activation of $\text{Ca}^{2+}$-permeable cation channels\textsuperscript{20} which subsequently leads to increase of cytosolic $\text{Ca}^{2+}$. The molecular identity of these cation channels has not been completely characterized but apparently involves TRPC6 channels\textsuperscript{22}. The cation channels are activated by prostaglandin E\textsubscript{2}, which is formed following exposure of erythrocytes to hyperosmotic shock\textsuperscript{19}. The channels are further activated by a wide variety of cell stressors, xenobiotics and endogenous mediators\textsuperscript{19}. $\text{Ca}^{2+}$ activates $\text{Ca}^{2+}$-sensitive K\textsuperscript{+} channels with exit of KCl and osmotically obliged water and thus cell shrinkage\textsuperscript{19,20}. An increase of cytosolic $\text{Ca}^{2+}$ is further followed by stimulation of cell membrane scrambling with exposure of phosphatidylserine (PS) at the cell surface\textsuperscript{19,20}. The $\text{Ca}^{2+}$ sensitivity of cell membrane scrambling is further enhanced by ceramide\textsuperscript{21}. PS-exposing cells are bound to macrophages, engulfed and degraded and thus cleared from circulating blood\textsuperscript{19,20,23–25}. To the best of our knowledge, the impact of $\text{G}_{\alpha_i}^2$ on erythrocyte survival and suicidal death has hitherto not been reported.

In the present study we explored whether the $\text{G}_{\alpha_i}^2$ isoform is expressed in erythrocytes and whether it participates in the regulation of erythrocyte survival. To this end, eryptosis was determined in erythrocytes from $\text{G}_{\alpha_i}^2$ knockout mice ($\text{G}_{\alpha_i}^2^{-/-}$) and their wild type littermates ($\text{G}_{\alpha_i}^2^{+/+}$).

**Results**

The present study addressed the impact of $\text{G}_{\alpha_i}^2$ on eryptosis in mice. To this end, experiments were performed in mice lacking functional $\text{G}_{\alpha_i}^2$ ($\text{G}_{\alpha_i}^2^{-/-}$) and corresponding wild type mice ($\text{G}_{\alpha_i}^2^{+/+}$). As shown in Fig. 1A, erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, mean corpuscular

![Figure 1. Blood parameters. Means ± SEM of erythrocyte count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and reticulocyte count (RTC) (A, n = 8), Ter119/CD71 positive cells (C, n = 6), plasma erythropoietin (EPO) levels (D, n = 3–4), leukocyte count (E, n = 8) and platelet count (F, n = 8) in $\text{G}_{\alpha_i}^2^{+/+}$ and $\text{G}_{\alpha_i}^2^{-/-}$ mice. *** (p < 0.001) significantly different from $\text{G}_{\alpha_i}^2^{+/+}$ mice. (B) May-Grünwald staining of erythrocytes from $\text{G}_{\alpha_i}^2^{+/+}$ and $\text{G}_{\alpha_i}^2^{-/-}$ mice.](https://www.nature.com/scientificreports/6/30925)
hemoglobin concentration and the percentage of reticulocytes were not significantly different between Gαi2+/− and Gαi2−/− mice. The mean corpuscular volume was slightly, but significantly larger in Gαi2−/− than in Gαi2+/− erythrocytes (41.1 ± 0.3 fl for Gαi2+/− mice versus 42.8 ± 0.2 fl for Gαi2−/− mice; n = 8, p < 0.001). Gαi2−/− erythrocytes are, thus, normochromic and moderately larger as compared to Gαi2+/− erythrocytes. May-Grünwald staining further revealed no apparent changes in erythrocyte shape from Gαi2−/− mice as compared to erythrocytes from Gαi2+/+ mice (Fig. 1B). The percentages of CD71/Ter119 positive cells were similar in Gαi2−/− and Gαi2+/+ mice suggesting similar patterns of dynamic erythrocyte maturation in vivo (Fig. 1C). Plasma erythropoietin concentrations were further similar in Gαi2−/− and Gαi2+/+ mice (Fig. 1D). Consistent with a previous report26, we observed leukocytosis in Gαi2−/− mice (Fig. 1E) which is attributed to increased production of proinflammatory cytokines in Gαi2−/− mice18. The platelet count in Gαi2−/− mice was, however, not significantly different from Gαi2+/+ mice (Fig. 1F).

Immunoblotting was employed to examine whether Gα2 is expressed in human and murine erythrocytes. To this end, erythrocytes from humans or from mice were isolated and purified. Equal amounts of protein lysates were immunoblotted. GAPDH served as a loading control. As depicted in Fig. 2A, the incubation with Gαi2+/+ mice was, however, not significantly different from Gαi2−/− mice (Fig. 1F).

Next, we explored whether Gαi2 deficiency influences erythrocyte survival. To this end, using annexin V binding, forward scatter and Fluo3 fluorescence in FACS analysis we analyzed erythrocyte cell membrane PS exposure, cell shrinkage and cytosolic Ca2+ activity, respectively. As depicted in Fig. 3A, freshly drawn and untreated erythrocytes were visualized using confocal microscopy and quantification of multiple fields showed a decreased ratio of annexin V binding to total cells (observed under transmission light) per field in Gαi2−/− erythrocytes (0.028 ± 0.007; n = 4) as compared to Gαi2+/+ erythrocytes (0.069 ± 0.007; n = 4). PS exposure was simultaneously quantified using FACS analysis (50,000 cells were quantified) and confirmed that in both freshly drawn blood (Fig. 3B,C) and following 12 h incubation in Ringer solution (Fig. 3D), the percentage of annexin V binding erythrocytes was significantly lower in Gαi2−/− mice than in Gαi2+/+ mice. Quantification of forward scatter showed that the cell volume was significantly larger in Gαi2−/− erythrocytes as compared to Gαi2+/+ erythrocytes (Fig. 4A,B). Both cell membrane PS exposure and cell shrinkage are influenced by cytosolic Ca2+ activity20. As shown in Fig. 4C,D, the percentage of Fluo3 positive erythrocytes was slightly but significantly lower in Gαi2−/− mice as compared to Gαi2+/+ mice. These data suggest an inhibitory effect of Gαi2 deficiency on eryptosis.

Further experiments then addressed the susceptibility of Gαi2-deficient erythrocytes to osmotic shock ex vivo, a pathophysiological cell stressor and a known stimulator of eryptosis. As illustrated in Fig. 5A,B, exposure of erythrocytes for 30 min to hyperosmotic Ringer (550 mM sucrose was added to the Ringer solution to reach the final osmolarity of 850 mOsM), significantly enhanced PS exposure, an effect, however, significantly blunted in Gαi2−/− erythrocytes as compared to Gαi2+/+ erythrocytes. Erythrocyte forward scatter was quantified to determine hyperosmotic shock-triggered cell shrinkage. As shown in Fig. 5C,D, forward scatter was significantly reduced by hyperosmotic shock in erythrocytes from both Gαi2−/− and Gαi2+/+ mice. The effect was significantly less pronounced in Gαi2−/− erythrocytes than in Gαi2+/+ erythrocytes.

To elucidate the mechanism contributing to the protective effect of Gαi2 deficiency against hyperosmotic shock-triggered eryptosis, we determined erythrocyte cytosolic Ca2+ activity following hyperosmotic shock. As shown in Fig. 6A,B, exposure of erythrocytes to hyperosmotic shock significantly enhanced the percentage of Fluo3 positive erythrocytes. The effect was, however, significantly blunted in Gαi2−/− erythrocytes as compared...
to Gai2+/+ erythrocytes. Further experiments explored the resistance of erythrocytes to a decline of extracellular osmolarity. As illustrated in Fig. 6C, the resistance of erythrocytes to graded decrease of osmolarity was significantly lower in Gai2+/+ than in Gai2−/− erythrocytes. Thus, Gai2 deficiency counteracts the sensitivity of erythrocytes to both hyper- and hypoosmotic shock.

Additional experiments explored whether erythrocyte Gai2 deficiency protects against ceramide-sensitive eryptosis. As shown in Fig. 7, treatment of erythrocytes from Gai2−/− and Gai2+/+ mice with C6 ceramide and bacterial sphingomyelinase significantly increased PS exposure, an effect, slightly, but significantly less pronounced in Gai2−/− erythrocytes as compared to Gai2+/+ erythrocytes. Thus, erythrocyte Gai2 deficiency has a subtle effect on ceramide-elicited eryptosis.

Discussion

The present observations disclose the expression of Gai2 in human and murine erythrocytes and further reveals that Gai2 deficiency confers partial protection against suicidal erythrocyte death or eryptosis. Our findings show that the percentage of eryptotic cells in circulating blood is significantly lower in Gai2−/− mice as in Gai2+/+ mice. Gai2−/− mice do not show overt changes in erythrocyte parameters such as erythrocyte count, hematocrit, hemoglobin concentration and reticulocyte count. The impact of Gai2 deficiency on erythrocytes is unmasked in the presence of pathophysiological cell stressors ex vivo such as hyperosmotic shock and following treatment with C6 ceramide and bacterial sphingomyelinase, whereby eryptosis is significantly less pronounced in Gai2−/− erythrocytes as compared to Gai2+/+ erythrocytes. Thus, erythrocyte Gai2 deficiency has a subtle effect on ceramide-elicited eryptosis.

Our data show that in the absence of stress, the difference between the percentage of PS-exposing erythrocytes in Gai2+/+ mice and Gai2−/− mice is subtle (~0.2%) yet statistically significant. Previous studies have shown that spontaneous PS exposure in freshly drawn erythrocytes from healthy wild-type mice of different strains does not exceed 1% of the total number of circulating erythrocytes. Thus, in transgenic mice which display a phenotype of reduced eryptosis, the percentage of PS-exposing circulating erythrocytes may be significantly lower than in wild-type mice despite relatively lower magnitudes of difference. Exposure of erythrocytes to hypertonic extracellular environment in vitro simulates the osmotic conditions encountered in the kidney medulla. In conditions

Figure 3. Phosphatidylserine externalization. (A) Confocal microscopy of annexin-V-fluorescence (right panels) and transmission light (middle and left panels) of erythrocytes from Gai2+/+ and Gai2−/− mice. Middle panels are amplified images of the area inside the squares of left panels. (B) Histogram (Blue: Gai2+/+, red: Gai2−/−) and means ± SEM of annexin-V-binding in erythrocytes freshly drawn (C, n = 24–40) or incubated 12 h in Ringer (D, n = 11–17). * (p < 0.05) significantly different from Gai2+/+ mice.
such as acute renal failure, erythrocytes may enter eryptosis due to their entrapment in the kidney medulla\(^21\). \(G_{\alpha i2}\) deficiency may blunt eryptosis and thus favorably influence the respective clinical condition. Our data show that, in addition to curtailing PS exposure, \(G_{\alpha i2}\)^{−/−} erythrocytes showed increased resistance to cell shrinkage following hyperosmotic shock. Accordingly, the mean corpuscular cell volume was significantly larger in \(G_{\alpha i2}\)^{−/−} erythrocytes. Along those lines, it is intriguing to speculate that \(G_{\alpha i2}\) influences cell volume regulatory ion channels in erythrocytes.

Mechanistically, hyperosmotic shock is a powerful stimulator of Ca\(^{2+}\) entry and ceramide formation in erythrocytes\(^20\). We observed that following hyperosmotic shock of erythrocytes, \(G_{\alpha i2}\) deficiency leads to subtle but significant decrease of cytosolic Ca\(^{2+}\) entry. On the other hand, \(G_{\alpha i2}\) may additionally mediate hyperosmotic shock-induced eryptosis by influencing ceramide signaling\(^21\). This is corroborated by our data showing a mitigating effect of \(G_{\alpha i2}\) deficiency on eryptosis triggered by either C6 ceramide or bacterial sphingomyelinase. Ceramide sensitizes erythrocytes to the eryptotic effect of enhanced Ca\(^{2+}\) concentration and may stimulate eryptosis without appreciable increase in cytosolic Ca\(^{2+}\) activity\(^27\). Ceramide further modifies the interaction of the erythrocyte membrane with the cytoskeleton thereby increasing membrane fragility\(^28\). As \(G_{\alpha i2}\) is an essential regulator for Ca\(^{2+}\) signaling in nucleated cells, it is possible that the inhibitory effect of \(G_{\alpha i2}\) deficiency on erythrocyte death is, at least in part, mediated by its influence on cytosolic Ca\(^{2+}\) activity.

Eryptosis is inhibited by catecholamines including dopamine\(^39\). Interestingly, dopamine-dependent signaling involves pertussis toxin-sensitive \(G_{\alpha i2}\)^{20}. Further signaling molecules that regulate the eryptosis machinery include AMPK\(^20\), p38 MAPK\(^31\), CK1\(\alpha\)\(^32\), PAK2\(^33\), PDK1\(^32\), MSK1/2\(^34\) and CDK4\(^35\). Eryptosis is triggered by a myriad of xenobiotics and endogenous substances\(^20,36–48\), and accelerated eryptosis contributes to the anemia associated with several clinical disorders\(^20\), including iron deficiency\(^49\), sepsis\(^50\), renal failure\(^51\), hepatic failure\(^52\), malignancy\(^53\), ageing\(^53\) and Wilson's disease\(^54\). Eryptotic erythrocytes adhere to the vascular wall\(^55\), and stimulate blood clotting\(^56\). Excessive eryptosis may thus interfere with microcirculation and participate in the vascular injury of metabolic syndrome\(^57\). Accordingly, \(G_{\alpha i2}\)^{−/−} mice may be particularly resistant to derangements of microcirculation following exposure to triggers of eryptosis. Moreover, eryptosis has been shown to influence the quality of stored erythrocytes\(^58\). Pharmacologically targeting \(G_{\alpha i2}\), at least in theory, may further provide new avenues in the treatment of conditions associated with anemia resulting from increased eryptosis\(^50\). On the other hand, \(G_{\alpha i2}\) modulation may serve as a novel target for the treatment of malaria, a condition where eryptosis plays a protective role in ameliorating parasitemia by expediting the clearance of pathogen-infected erythrocytes\(^50\).

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**Figure 4. Cell shrinkage and cytosolic Ca\(^{2+}\)-activity.** Histogram (A,C; Blue: \(G_{\alpha i2}\)^{+/+}, red: \(G_{\alpha i2}\)^{−/−}) and means ± SEM of forward scatter (B, \(n = 21–33\)) and percentage of Fluo3 positive erythrocytes (D, \(n = 8–16\)). ***,***(\(p < 0.05\), \(p < 0.01\)) significantly different from \(G_{\alpha i2}\)^{+/+} mice.
In conclusion, the G-protein subunit Gαi2 is expressed in human and murine erythrocytes and participates in the regulation of erythrocyte suicide.

Materials and Methods

Mice. Experiments were performed in Gαi2 knockout mice (Gαi2−/−) and their wild type littermates (Gαi2+/+) of 6–9 weeks of age. The mice were generated and initially characterized on a SV129 background.18 Mice were backcrossed on a C57BL6 background and kept under specified pathogen-free (SPF) environment in individually ventilated cages (IVC) to prolong life expectancy. All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by local government authorities (Regierungspräsidium Tübingen).

Blood count, incubation and solutions. For all experiments except for the blood count, heparin blood was retrieved from the retrobulbar plexus of mice. For the blood count, EDTA blood was analyzed using an electronic hematology particle counter (type MDM 905 from Medical Diagnostics Marx; Butzbach, Germany) equipped with a photometric unit for haemoglobin determination. Plasma erythropoietin levels were determined using an immunoassay kit according to the manufacturer’s instructions (R&D Systems, Wiesbaden, Germany). Murine erythrocytes were isolated by being washed two times with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 MgSO4, and 32 HEPES/NaOH (pH 7.4), 5 glucose, and 1 CaCl2. Where indicated, sucrose (550 mM), C6 ceramide (50 μM; Sigma) or bacterial sphingomyelinase (0.01 U/ml; Sigma) were added to the Ringer solution. May–Grünwald staining was used to examine changes in erythrocyte shape. Briefly, 20 μl of erythrocytes were smeared and fixed using methanol onto a glass slide, and stained with 5% Giemsa Azur-Eosin (Merck Millipore, Germany) in phosphate-buffered saline (in mM: 1.05 KH2PO4, 2.97 Na2HPO4, 155.2 NaCl) for 20 min. Subsequently, images were taken on a Nikon Diaphot 300 Microscope (Nikon Instruments, Germany).

Reticulocyte count and markers of erythrocyte maturation. For determination of the reticulocyte count EDTA-whole blood (2.5 μl) was added to 500 μl Retic-COUNT (Thiazole orange) reagent from Becton Dickinson. Samples were stained for 30 min at room temperature, and flow cytometry was performed according to the manufacturer’s instructions. Forward scatter (FSC), side scatter (SSC), and thiazole orange-fluorescence intensity...
(in FL-1) of the blood cells were determined. The number of Retic-COUNT positive reticulocytes was expressed as the percentage of the total gated erythrocyte populations. Gating of erythrocytes was achieved by analysis of FSC vs. SSC dot plots using CellQuest software. To further examine the dynamic maturation of erythrocytes in vivo, erythrocytes were double stained with CD71 (1:12.5; BD Biosciences), and Ter119 (1:250; BD Biosciences). Ter119 and CD71 positive cells were quantified by analyzing the upper right quadrant of an FL1 versus FL2 dot plot.

Phosphatidylserine exposure and forward scatter. After incubation, erythrocytes were washed once in Ringer solution containing 5 mM CaCl₂. The cells were then stained with annexin-V-FITC (1:250 dilution; Immunotools, Friesoythe, Germany) at a 1:50 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur; BD). Cells were analyzed by forward scatter, and annexin V fluorescence intensity was measured in fluorescence channel FL-1 with excitation and emission wavelengths of 488 nm and 530 nm, respectively, on a FACS Calibur (BD, Heidelberg, Germany) as described previously. Where indicated, spontaneous PS exposure and forward scatter were determined by addition of 2 μl of freshly drawn erythrocytes in 500 μl Ringer solution containing 5 mM CaCl₂ and annexin-V-FITC. Raw data for annexin V positive erythrocytes was collected by a primary gating of the erythrocyte population on FSC vs. SSC dot plots and, subsequently, by setting an arbitrary marker at the base of the cell population on an FL1 histogram. The cell population plotted on the left of the arbitrary marker was considered positive for annexin V binding.

Estimation of intracellular Ca²⁺. For measurement of intracellular Ca²⁺, 50 μl erythrocyte suspension was washed in Ringer solution and then loaded with Fluo-3/AM (Biotrend, Köln, Germany) at a 1:500 dilution. After 30 min, the cells were washed twice in Ringer solution and then incubated with 5 mM CaCl₂ for 30 min before being resuspended in 500 μl Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 500 μl Ringer solution containing 5 mM CaCl₂. The fluorescence intensity was measured in the fluorescence channel FL-1 in a FACS analysis. Where indicated, spontaneous intracellular Ca²⁺ was determined by addition of 2 μl of freshly drawn erythrocytes in 500 μl Ringer solution containing 5 mM CaCl₂ as well as Fluo3/AM. Fluo3 positive cells were plotted using an FL1 histogram similar to the analysis of annexin V positive cells.

Determination of the osmotic resistance. Two microliters of blood were added to 200 μl of PBS solutions with decreasing osmolarity. After centrifugation for 5 min at 3000 rpm, the supernatant was transferred to a
96-well plate, and the absorption at 405 nm was determined as a measure of hemolysis. Absorption of the supernatant of erythrocytes lysed in pure distilled water was defined as 100% hemolysis.

Immunoblotting. To examine the expression of Gαi2 in human or murine erythrocytes, 150 μl erythrocyte pellet was lysed in 50 μl of 20 mM HEPES/NaOH (pH 7.4). Ghost membranes were pelleted (15,000 g for 20 min at 4 °C) and lysed in 200 μl lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.5% SDS; 1 mM NaF; 1 mM Na3VO4; and 0.4% β-mercaptoethanol) containing protease inhibitor cocktail (Sigma, Schnelldorf, Germany). Triton X-100, a non-ionic detergent, was used in erythrocyte ghost preparation due to its effective solubilization power and a relatively mild effect on membrane-bound enzymes.60 In all cases, 60 μg of protein was solubilized in Laemmli sample buffer at 95 °C for 5 min and resolved by pre-casted 10% SDS-PAGE gel (Invitrogen, Karlsruhe, Germany). For immunoblotting, proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 1 h. Then, the membrane was incubated with anti-G-protein alpha inhibitor 2 antibody (1:5000; 40 kDa; Abcam Cat# ab157204) at 4 °C overnight. After being washed (in TBS-0.10% Tween 20) and subsequently blocked, the blots were incubated with secondary anti-rabbit antibody (1:2000; Cell Signaling) for 1 h at room temperature. After being washed, the antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany).

Confocal microscopy and immunofluorescence. For the visualization of eryptotic erythrocytes, 4 μl of erythrocytes, incubated in the respective experimental solutions, were stained with FITC-conjugated annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 μl Ringer solution containing 5 mM CaCl2. Then, the erythrocytes were washed twice and finally resuspended in 50 μl of Ringer solution containing 5 mM CaCl2. Twenty μl were mounted with Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) onto a glass slide and covered with a coverslip. Sections were analyzed using a Leica TCS-SP / Leica DM RB confocal laser scanning microscope. Images were processed with Leica Confocal Software LCS (Version 2.61).

Statistics. Data are expressed as arithmetic means ± SEM, and statistical analysis was made using ANOVA or t-test, as appropriate. n denotes the number of different erythrocyte specimens studied.

Figure 7. Effect of C6-ceramide and bacterial sphingomyelinase on phosphatidylserine externalization. Histograms (A,C: Blue: Gαi2+/−, red: Gαi2−/−) and means ± SEM of annexin-V-binding following exposure to C6-ceramide (A,B; 50 μM, 12 h; n = 11–17) or bacterial sphingomyelinase (C,D; 0.01 U/ml 24 h; n = 7–16). *p (p < 0.001) significantly different from Control. *p (p < 0.05) from Gαi2+/−.
References

1. Carmichael, C. Y. & Wainford, R. D. Brain Galphai 2 -subunit proteins and the prevention of salt sensitive hypertension. *Front Physiol* 6, 233 (2015).

2. Wetscherek, N. & Offermanns, S. Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85, 1159–1204 (2005).

3. Simon, M. I., Strathmann, M. P. & Gautam, N. Diversity of G proteins in signal transduction. *Science* 252, 802–808 (1991).

4. Devanathan, V. et al. Platelet Gi protein Galphai2 is an essential mediator of thrombo-inflammatory organ damage in mice. *Proc Natl Acad Sci USA* 112, 6491–6496 (2015).

5. Pero, R. S. et al. Galphai2-mediated signaling events in the endothelium are involved in controlling leukocyte extravasation. *Proc Natl Acad Sci USA* 104, 4371–4376 (2007).

6. Ngai, J., Innigerdingen, M., Berge, T. & Tasken, K. Interplay between the heterotrimeric G-protein subunits Galphaq and Galphai2 sets the threshold for chemotaxis and TCR activation. *BMC Immunol* 10, 27 (2009).

7. Zarbock, A., Deem, T. L., Burcin, T. L. & Ley, K. Galphai2 is required for chemokin-induced neutrophil arrest. *Blood* 110, 3773–3779 (2007).

8. Minetti, G. C. et al. Galphai2 signaling is required for skeletal muscle growth, regeneration, and satellite cell proliferation and differentiation. *Mol Cell Biol* 34, 619–630 (2014).

9. Lopez-Aranda, M. F. et al. Activation of caspase-3 pathway by expression of galphai2 protein in BHK cells. *Neurosci Lett* 439, 37–41 (2008).

10. Meyeon, J. & al. Close spatio-association of the transient receptor potential canonical 4 (TRPC4) channel with Galphai in TRPC4 activation pathway. *Am J Physiol Cell Physiol* 308, C879–C889 (2015).

11. Dizaye, S. et al. Galphai2- and Galphai3-specific regulation of voltage-dependent L-type calcium channels in cardiomyocytes. *PLoS One* 6, e24979 (2011).

12. Dizaye, S., Kakei, M. & Yata, T. Ghrelin uses Galphai2 and activates voltage-dependent K+-channels to attenuate glucose-induced Ca2+ signaling and insulin release in islet beta-cells: novel signal transduction of ghrelin. *Diabetes* 56, 2319–2327 (2007).

13. Cao, C. et al. Galphai(1) and Galphai(3) are required for epidermal growth factor-mediated activation of the Akt-mTORC1 pathway. *Sci Signal* 2, ra17 (2009).

14. Duronio, V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. *Biochem J* 415, 333–344 (2008).

15. Leon, J. P. et al. Activation of TRPC3/ETA by Galphai subunit increases Ca2+ selectivity and controls neurite morphogenesis in cultured hippocampal neuron. *Calcium* 54, 307–319 (2013).

16. Singh, V. R., Raghuwanshi, S. K., Smith, N., Rivers, E. J. & Richardson, R. M. G Protein-coupled receptor kinase-6 interacts with activator of G protein signaling-3 to regulate CXCR2-mediated cellular functions. *J Immunol* 192, 2186–2194 (2014).

17. Zuberi, Z. et al. Absence of the inhibitory G-protein Galphai2 predisposes to ventricular cardiac arrhythmia. *Circ Arrhythm Electrophysiol* 3, 391–400 (2010).

18. Rudolph, U. et al. Ulcerative colitis and adenocarcinoma of the colon in Galphai2-deficient mice. *Nat Genet* 10, 143–150 (1995).

19. Lang, F. & Qadri, S. M. Mechanisms and significance of erythropoiesis, the suicidal death of erythrocytes. *Blood Purif* 33, 125–130 (2012).

20. Lang, F., Qadri, S. M. & Lang, F. Killing me softly-suicidal erythrocyte death. *Cell Physiol Biochem* 33, 528–549 (2014).

21. Lang, K. S. et al. Involvement of ceramide in hypersometric shock-induced death of erythrocytes. *Cell Death Differ* 11, 231–243 (2004).

22. Foller, M. et al. TRPC6 contributes to the Ca(2+)-leak of human erythrocytes. *Cell Physiol Biochem* 21, 183–192 (2008).

23. Zidova, Z. et al. DMT1-mutant erythrocytes have shortened life span, accelerated glycolysis and increased oxidative stress. *Cell Physiol Biochem* 22, 2221–2231 (2014).

24. Qadri, S. M. et al. Enhanced suicidal erythrocyte death in mice carrying a loss-of-function mutation of the adenomatous polyposis coli gene. *J Cell Mol Med* 16, 1085–1093 (2012).

25. Foller, M. et al. Functional significance of glutamate-cysteine ligase modifier for erythrocyte survival in vitro and in vivo. *Cell Death Differ* 20, 1350–1358 (2013).

26. Ohman, L., Franzen, L., Rudolph, U., Harriman, G. R. & Hultgren, H. E. Immune activation in the intestinal mucosa before the onset of colitis in Galphai2-deficient mice. *Scand J Immunol* 52, 80–90 (2000).

27. Lang, E., Bissinger, R., Gullbins, E. & Lang, F. Ceramide in the regulation of eryptosis, the suicidal erythrocyte death. *Apoptosis* 20, 758–767 (2015).

28. Dinkla, S. et al. Functional consequences of sphingomyelinase-induced changes in erythrocyte membrane structure. *Cell Death Dis* 3, e410 (2012).

29. Lang, P. A. et al. Inhibition of erythrocyte “apoptosis” by catecholamines. *Naunyn Schmiedebergs Arch Pharmacol* 372, 228–235 (2005).

30. Neve, K. A., Seams, J. K. & Trantham-Davidson, H. Dopamine receptor signaling. *J Recept Signal Transduct Res* 24, 165–205 (2004).

31. Gatidis, S. et al. p38 MAPK activation and function following osmotic shock of erythrocytes. *Cell Physiol Biochem* 28, 1279–1286 (2011).

32. Zelenak, C. et al. Protein kinase CK1alpalpha regulates erythrocyte survival. *Cell Physiol Biochem* 29, 171–180 (2012).

33. Zelenak, C. et al. Proteme analysis of erythrocytes lacking AMP-activated protein kinase reveals a role of PAK2 kinase in erythropoiesis. *J Proteome Res* 10, 1690–1697 (2011).

34. Lang, E. et al. Accelerated apoptotic death and in vivo turnover of erythrocytes in mice lacking functional mitogen- and stress-activated kinase MSK1/2. *Sci Rep* 5, 17316 (2015).

35. Lang, E. et al. Impact of Cyclin-Dependent Kinase CDK4 Inhibition on Erythropoiesis. *Cell Physiol Biochem* 37, 1178–1186 (2015).

36. Arnold, M., Bissinger, R. & Lang, F. Mitoxantrone-induced suicidal erythrocyte death. *Cell Physiol Biochem* 34, 1756–1767 (2014).

37. Bissinger, R., Fischer, S., Jilani, K. & Lang, F. Stimulation of erythrocyte death by phloretin. *Cell Physiol Biochem* 34, 2256–2265 (2014).

38. Bissinger, R., Lupescu, A., Zelenak, C., Jilani, K. & Lang, F. Stimulation of eryptosis by cryptotanshinone. *Cell Physiol Biochem* 34, 432–442 (2014).

39. Zhang, R. et al. Involvement of calcium, reactive oxygen species, and ATP in hexavalent chromium-induced damage in red blood cells. *Cell Physiol Biochem* 34, 1780–1791 (2014).

40. Tesoriere, L. et al. Oxysterol mixture in hypercholesterolemia-relevant proportion causes oxidative stress-dependent erythropoiesis. *Cell Physiol Biochem* 34, 1075–1089 (2014).

41. Risso, A., Ciana, A., Achilli, C. & Minetti, G. Survival and senescence of human young red cells in vitro. *Cell Physiol Biochem* 34, 1038–1049 (2014).

42. Lupescu, A., Bissinger, R., Warsi, J., Jilani, K. & Lang, F. Stimulation of erythrocyte membrane scrambling by gedunin. *Cell Physiol Biochem* 33, 1838–1848 (2014).

43. Faggio, C., Alzoubi, K., Calabro, S. & Lang, F. Stimulation of suicidal erythrocyte death by PRIMA-1. *Cell Physiol Biochem* 35, 529–540 (2015).

44. Peter, T. et al. Programmed erythrocyte death following in vitro Treosulfan treatment. *Cell Physiol Biochem* 35, 1372–1380 (2015).

45. Officiocio, A., Alzoubi, K., Manna, C. & Lang, F. Clofazimine Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 37, 331–341 (2015).
46. Fazio, A., Briglia, M., Faggio, C., Alzoubi, K. & Lang, F. Stimulation of Suicidal Erythrocyte Death by Garcinol. *Cell Physiol Biochem* 37, 805–815 (2015).

47. Lang, E. *et al.* Vitamin D-Rich Diet in Mice Modulates Erythrocyte Survival. *Kidney Blood Press Res* 40, 403–412 (2015).

48. Ran, Q. *et al.* Eryptosis Indices as a Novel Predictive Parameter for Biocompatibility of Fe3O4 Magnetic Nanoparticles on Erythrocytes. *Sci. Rep* 5, 16209 (2015).

49. Kempe, D. S. *et al.* Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J* 20, 368–370 (2006).

50. Kempe, D. S. *et al.* Suicidal erythrocyte death in sepsis. *J. Mol. Med. (Berl)* 85, 273–281 (2007).

51. Abed, M. *et al.* Conjugated bilirubin triggers anemia by inducing erythrocyte death. *Hepatology* 61, 275–284 (2015).

52. Kempe, D. S. *et al.* Enhanced suicidal erythrocyte death contributing to anemia in the elderly. *Cell Physiol Biochem* 36, 773–783 (2015).

53. Lang, P. A. *et al.* Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nat. Med* 13, 164–170 (2007).

54. Borst, O. *et al.* Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. *Am. J. Physiol Cell Physiol* 302, C644–C651 (2012).

55. Chung, S. M. *et al.* Lysephosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. *Arterioscler. Thromb. Vasc. Biol* 27, 414–421 (2007).

56. Zappulla, D. Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO2 increases? *J. Cardiometab. Syndr* 3, 30–34 (2008).

57. Helenius, A. & Simons, K. Solubilization of membranes by detergents. *Biochim Biophys Acta* 415, 29–79 (1975).

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Author Contributions
F.L. and S.M.Q. designed the project and wrote the main manuscript text. R.B., E.L., M.G., Y.S., C.Z., B.F., S.H., H.C., H.F., A.T.U., G.L., R.R., G.L., M.S., A.F.M., A.L., L.B. and S.M.Q. performed the acquisition, analysis and/or interpretation of data. R.B., M.G., Y.S., B.F. and S.M.Q. prepared the figures. All authors have read and reviewed the manuscript and approved the final version.

Additional Information
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