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

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Putative functions of the heterotrimeric G-protein subunit $G\alpha i2$ -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 $G\alpha i2$ is expressed in both murine and human erythrocytes and further examined the survival of erythrocytes drawn from $G\alpha i2$ -deficient mice ($G\alpha i2^{-/-}$) and corresponding wild-type mice ($G\alpha i2^{+/+}$). Our data show that plasma erythropoietin levels, erythrocyte maturation markers, erythrocyte counts, hematocrit and hemoglobin concentration were similar in $G\alpha i2^{-/-}$ and $G\alpha i2^{+/+}$ mice but the mean corpuscular volume was significantly larger in $G\alpha i2^{-/-}$ mice. Spontaneous PS exposure of circulating $G\alpha i2^{-/-}$ erythrocytes was significantly lower than that of circulating $G\alpha i2^{+/+}$ erythrocytes. PS exposure was significantly lower in $G\alpha i2^{-/-}$ than in $G\alpha i2^{+/+}$ erythrocytes following *ex vivo* exposure to hyperosmotic shock, bacterial sphingomyelinase or C6 ceramide. Erythrocyte $G\alpha i2$ deficiency further attenuated hyperosmotic shock-induced increase of cytosolic Ca^{2+} activity and cell shrinkage. Moreover, $G\alpha i2^{-/-}$ erythrocytes were more resistant to osmosensitive hemolysis as compared to $G\alpha i2^{+/+}$ erythrocytes. In conclusion, $G\alpha i2$ 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 $G\alpha i$ is defined by their ability to downregulate cAMP levels by inhibition of adenylyl cyclase^{2,3}. The closely-related $G\alpha$ members $G\alpha i1$, $G\alpha i2$, and $G\alpha i3$, sharing 85–95% of their amino acid sequence identity, are characterized by their sensitivity to pertussis toxin^{2,3}. $G\alpha i2$, the quantitatively predominant $G\alpha i$ isoform, is a decisive regulator of leukocyte, endothelial and platelet functions^{4–7}. Further putative roles of $G\alpha i2$ signaling include ion channel regulation, cell differentiation, proliferation and apoptosis^{8–12}. Effector kinases of G-protein signaling include phosphoinositide 3-kinases¹³, which are known to be involved in the regulation of apoptosis¹⁴. $G\alpha i2$ further influences Ca^{2+} signaling in nucleated cells by the activation of TRPC4 channels which, in turn, increases Ca^{2+} influx¹⁵. In cardiomyocytes, $G\alpha i2$ has been shown to modulate the activity of L-type

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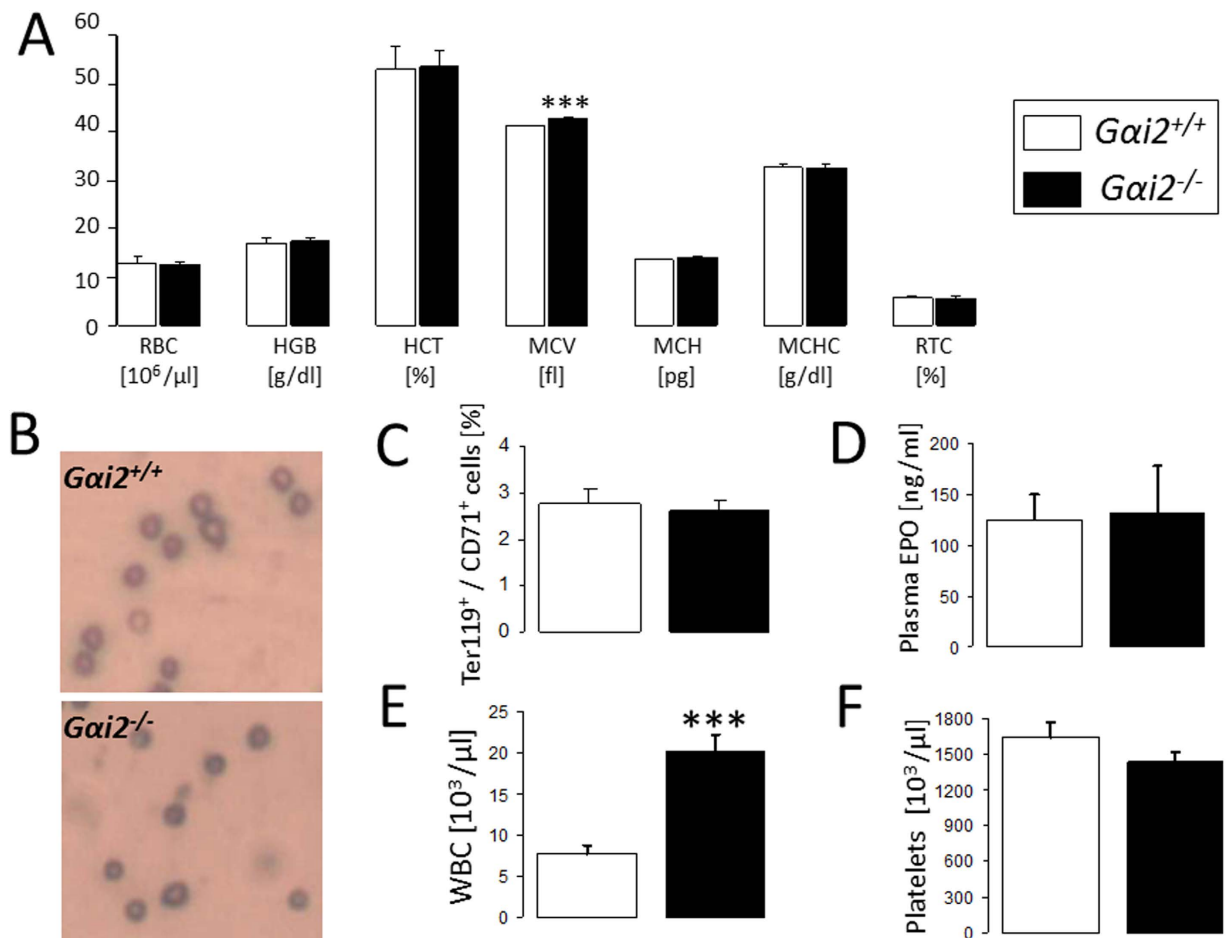


Figure 1. Blood parameters. Means \pm SEM of erythrocyte count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and reticulocyte count (RTC) (A, $n = 8$), Ter119/CD71 positive cells (C, $n = 6$), plasma erythropoietin (EPO) levels (D, $n = 3-4$), leukocyte count (E, $n = 8$) and platelet count (F, $n = 8$) in *Gai2*^{+/+} and *Gai2*^{-/-} mice. *** ($p < 0.001$) significantly different from *Gai2*^{+/+} mice. (B) May-Grünwald staining of erythrocytes from *Gai2*^{+/+} and *Gai2*^{-/-} mice.

voltage-dependent Ca^{2+} -channels¹¹. Furthermore, *Gai2* is a powerful regulator of cytosolic Ca^{2+} activity in islet beta cells¹² and neutrophils¹⁶, thus, regulating a variety of Ca^{2+} -dependent cell functions. Phenotypically, *Gai2* 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^{4,17,18}.

Similar to nucleated cells, erythrocytes may undergo suicidal death or eryptosis^{19,20}, which, similar to apoptosis, is triggered by osmotic shock and characterized by cell shrinkage and cell membrane scrambling^{20,21}. Eryptosis may be triggered by activation of Ca^{2+} -permeable cation channels²⁰ which subsequently leads to increase of cytosolic Ca^{2+} . The molecular identity of these cation channels has not been completely characterized but apparently involves TRPC6 channels²². The cation channels are activated by prostaglandin E_2 , which is formed following exposure of erythrocytes to hyperosmotic shock¹⁹. The channels are further activated by a wide variety of cell stressors, xenobiotics and endogenous mediators¹⁹. Ca^{2+} activates Ca^{2+} -sensitive K^+ channels with exit of KCl and osmotically obliged water and thus cell shrinkage^{19,20}. An increase of cytosolic Ca^{2+} is further followed by stimulation of cell membrane scrambling with exposure of phosphatidylserine (PS) at the cell surface^{19,20}. The Ca^{2+} sensitivity of cell membrane scrambling is further enhanced by ceramide²¹. PS-exposing cells are bound to macrophages, engulfed and degraded and thus cleared from circulating blood^{19,20,23-25}. To the best of our knowledge, the impact of *Gai2* on erythrocyte survival and suicidal death has hitherto not been reported.

In the present study we explored whether the *Gai2* isoform is expressed in erythrocytes and whether it participates in the regulation of erythrocyte survival. To this end, eryptosis was determined in erythrocytes from *Gai2* knockout mice (*Gai2*^{-/-}) and their wild type littermates (*Gai2*^{+/+}).

Results

The present study addressed the impact of *Gai2* on eryptosis in mice. To this end, experiments were performed in mice lacking functional *Gai2* (*Gai2*^{-/-}) and corresponding wild type mice (*Gai2*^{+/+}). As shown in Fig. 1A, erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, mean corpuscular

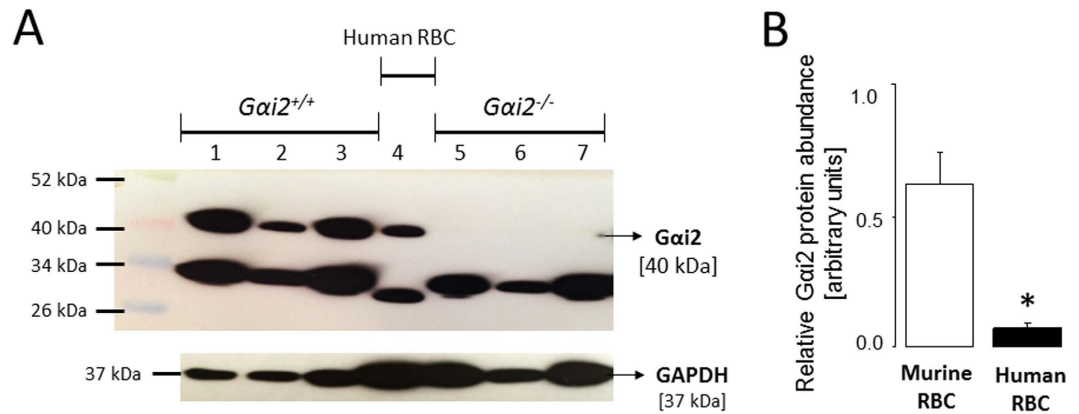


Figure 2. *Gai2* expression. (A) Western blots showing *Gai2* (40 kDa) and GAPDH (37 kDa) expression in erythrocytes from *Gai2*^{+/+} (bands 1, 2 and 3) or *Gai2*^{-/-} (bands 5, 6 and 7) mice and humans (band 4) in whole blood (bands 1 and 5), diluted whole blood (bands 2 and 6; 1:3.7 dilution) and purified erythrocytes (bands 3 and 7). (B) Means \pm SEM of *Gai2* abundance in murine and human erythrocytes relative to the loading control GAPDH (n = 3).

hemoglobin concentration and the percentage of reticulocytes were not significantly different between *Gai2*^{-/-} and *Gai2*^{+/+} mice. The mean corpuscular volume was slightly, but significantly larger in *Gai2*^{-/-} than in *Gai2*^{+/+} erythrocytes (41.1 ± 0.3 fl for *Gai2*^{+/+} mice versus 42.8 ± 0.2 fl for *Gai2*^{-/-} mice; n = 8, p < 0.001). *Gai2*^{-/-} erythrocytes are, thus, normochromic and moderately larger as compared to *Gai2*^{+/+} erythrocytes. May-Grünwald staining further revealed no apparent changes in erythrocyte shape from *Gai2*^{-/-} mice as compared to erythrocytes from *Gai2*^{+/+} mice (Fig. 1B). The percentages of CD71/Ter119 positive cells were similar in *Gai2*^{-/-} and *Gai2*^{+/+} mice suggesting similar patterns of dynamic erythrocyte maturation *in vivo* (Fig. 1C). Plasma erythropoietin concentrations were further similar in *Gai2*^{-/-} and *Gai2*^{+/+} mice (Fig. 1D). Consistent with a previous report²⁶, we observed leukocytosis in *Gai2*^{-/-} mice (Fig. 1E) which is attributed to increased production of proinflammatory cytokines in *Gai2*^{-/-} mice¹⁸. The platelet count in *Gai2*^{-/-} mice was, however, not significantly different from *Gai2*^{+/+} mice (Fig. 1F).

Immunoblotting was employed to examine whether *Gai2* 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 *Gai2* specific antibodies yielded a single band of 40 kDa in human erythrocytes as well as erythrocytes from *Gai2*^{+/+} mice, but not in erythrocytes from *Gai2*^{-/-} mice. The bands appearing below 40 kDa are presumably the result of non-specific antibody binding. Densitometry analysis revealed that *Gai2* protein is significantly more abundant in mouse erythrocytes as compared to human erythrocytes (Fig. 2B). Thus, *Gai2* is expressed in both human and murine erythrocytes.

Next, we explored whether *Gai2* 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 cells to total cells (observed under transmission light) per field in *Gai2*^{-/-} erythrocytes (0.028 ± 0.007 ; n = 4) as compared to *Gai2*^{+/+} 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 *Gai2*^{-/-} mice than in *Gai2*^{+/+} mice. Quantification of forward scatter showed that the cell volume was significantly larger in *Gai2*^{-/-} erythrocytes as compared to *Gai2*^{+/+} 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 *Gai2*^{-/-} mice as compared to *Gai2*^{+/+} mice. These data suggest an inhibitory effect of *Gai2* deficiency on eryptosis.

Further experiments then addressed the susceptibility of *Gai2*-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 *Gai2*^{-/-} erythrocytes as compared to *Gai2*^{+/+} 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 *Gai2*^{-/-} and *Gai2*^{+/+} mice. The effect was significantly less pronounced in *Gai2*^{-/-} erythrocytes than in *Gai2*^{+/+} erythrocytes.

To elucidate the mechanism contributing to the protective effect of *Gai2* 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 *Gai2*^{-/-} erythrocytes as compared

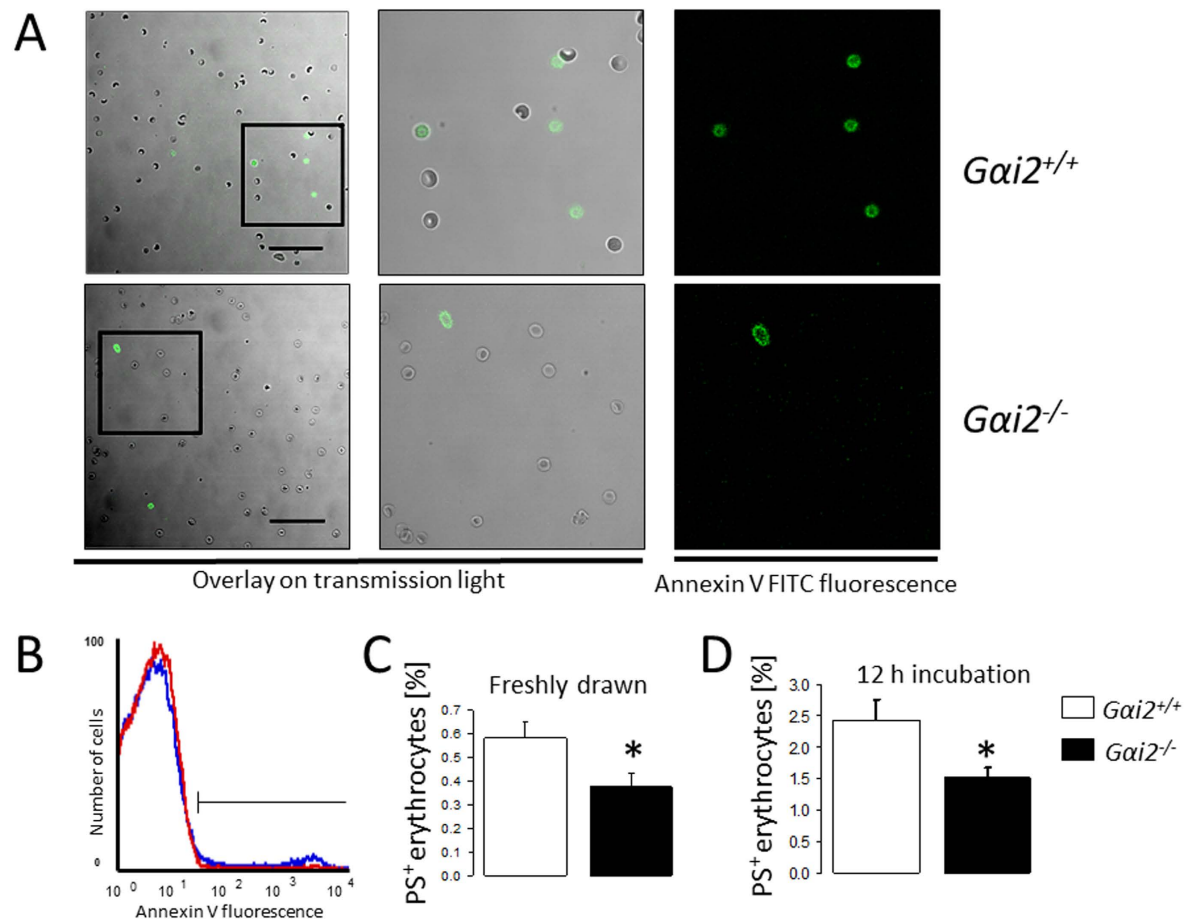


Figure 3. Phosphatidylserine externalization. (A) Confocal microscopy of annexin-V-fluorescence (right panels) and transmission light (middle and left panels) of erythrocytes from *Gai2*^{+/+} and *Gai2*^{-/-} mice. Middle panels are amplified images of the area inside the squares of left panels. (B) Histogram (Blue: *Gai2*^{+/+}, red: *Gai2*^{-/-}) and means \pm 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.

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.

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%¹⁹ 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 medulla²⁰. In conditions

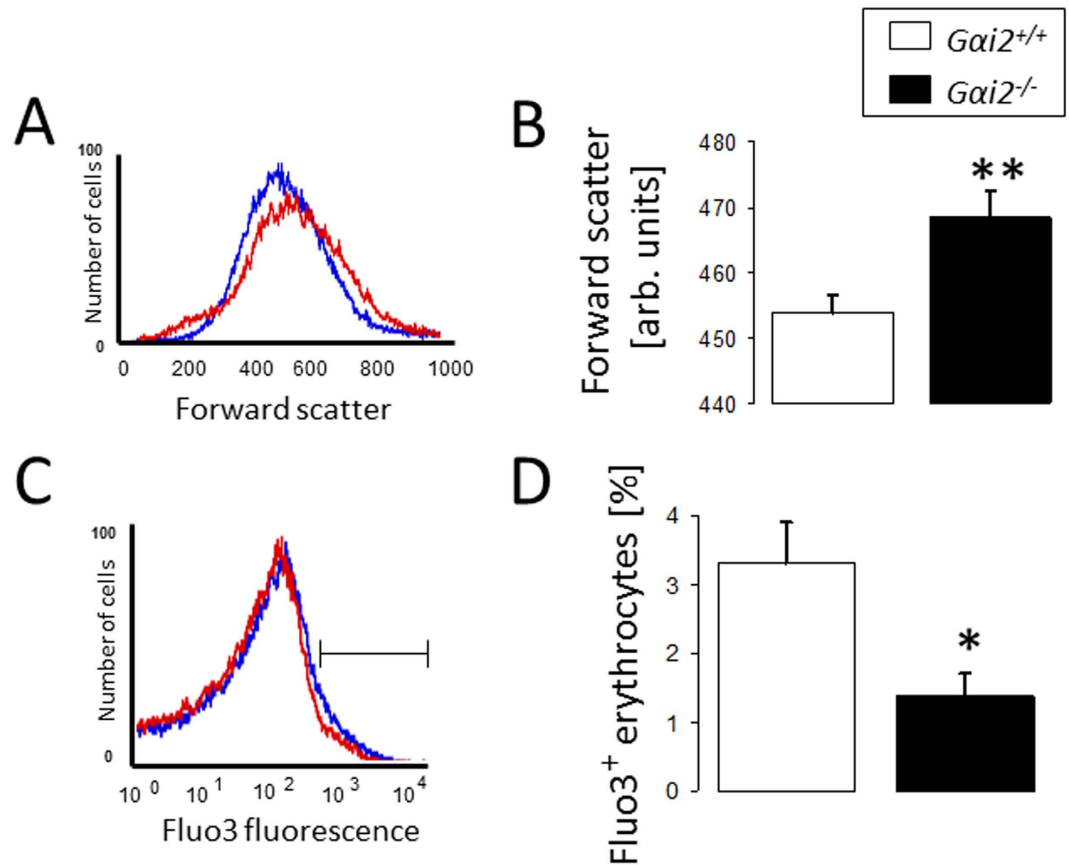


Figure 4. Cell shrinkage and cytosolic Ca²⁺-activity. Histogram (A,C; Blue: *Gai2*^{+/+}, red: *Gai2*^{-/-}) and means \pm SEM of forward scatter (B, n = 21–33) and percentage of Fluo3 positive erythrocytes (D, n = 8–16). ***(p < 0.05, p < 0.01) significantly different from *Gai2*^{+/+} mice.

such as acute renal failure, erythrocytes may enter eryptosis due to their entrapment in the kidney medulla²¹. *Gai2* deficiency may blunt eryptosis and thus favorably influence the respective clinical condition. Our data show that, in addition to curtailing PS exposure, *Gai2*^{-/-} erythrocytes showed increased resistance to cell shrinkage following hyperosmotic shock. Accordingly, the mean corpuscular cell volume was significantly larger in *Gai2*^{-/-} erythrocytes. Along those lines, it is intriguing to speculate that *Gai2* influences cell volume regulatory ion channels in erythrocytes.

Mechanistically, hyperosmotic shock is a powerful stimulator of Ca²⁺ entry and ceramide formation in erythrocytes²⁰. We observed that following hyperosmotic shock of erythrocytes, *Gai2* deficiency leads to subtle but significant decrease of cytosolic Ca²⁺ entry. On the other hand, *Gai2* may additionally mediate hyperosmotic shock-induced eryptosis by influencing ceramide signaling²¹. This is corroborated by our data showing a mitigating effect of *Gai2* deficiency on eryptosis triggered by either C6 ceramide or bacterial sphingomyelinase. Ceramide sensitizes erythrocytes to the eryptotic effect of enhanced Ca²⁺ concentration and may stimulate eryptosis without appreciable increase in cytosolic Ca²⁺ activity²⁷. Ceramide further modifies the interaction of the erythrocyte membrane with the cytoskeleton thereby increasing membrane fragility²⁸. As *Gai2* is an essential regulator for Ca²⁺ signaling in nucleated cells, it is possible that the inhibitory effect of *Gai2* deficiency on erythrocyte death is, at least in part, mediated by its influence on cytosolic Ca²⁺ activity.

Eryptosis is inhibited by catecholamines including dopamine²⁹. Interestingly, dopamine-dependent signaling involves pertussis toxin-sensitive *Gai2*³⁰. Further signaling molecules that regulate the eryptosis machinery include AMPK²⁰, p38 MAPK³¹, CK1 α ³², PAK2³³, PDK1²⁰, MSK1/2³⁴ and CDK4³⁵. 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 disorders²⁰, including iron deficiency⁴⁹, sepsis⁵⁰, renal failure⁵¹, hepatic failure⁵², malignancy²⁴, ageing⁵³ and Wilson's disease⁵⁴. Eryptotic erythrocytes adhere to the vascular wall⁵⁵, and stimulate blood clotting⁵⁶. Excessive eryptosis may thus interfere with microcirculation and participate in the vascular injury of metabolic syndrome⁵⁷. Accordingly, *Gai2*^{-/-} 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 erythrocytes⁵⁸. Pharmacologically targeting *Gai2*, at least in theory, may further provide new avenues in the treatment of conditions associated with anemia resulting from increased eryptosis²⁰. On the other hand, *Gai2* 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 erythrocytes²⁰.

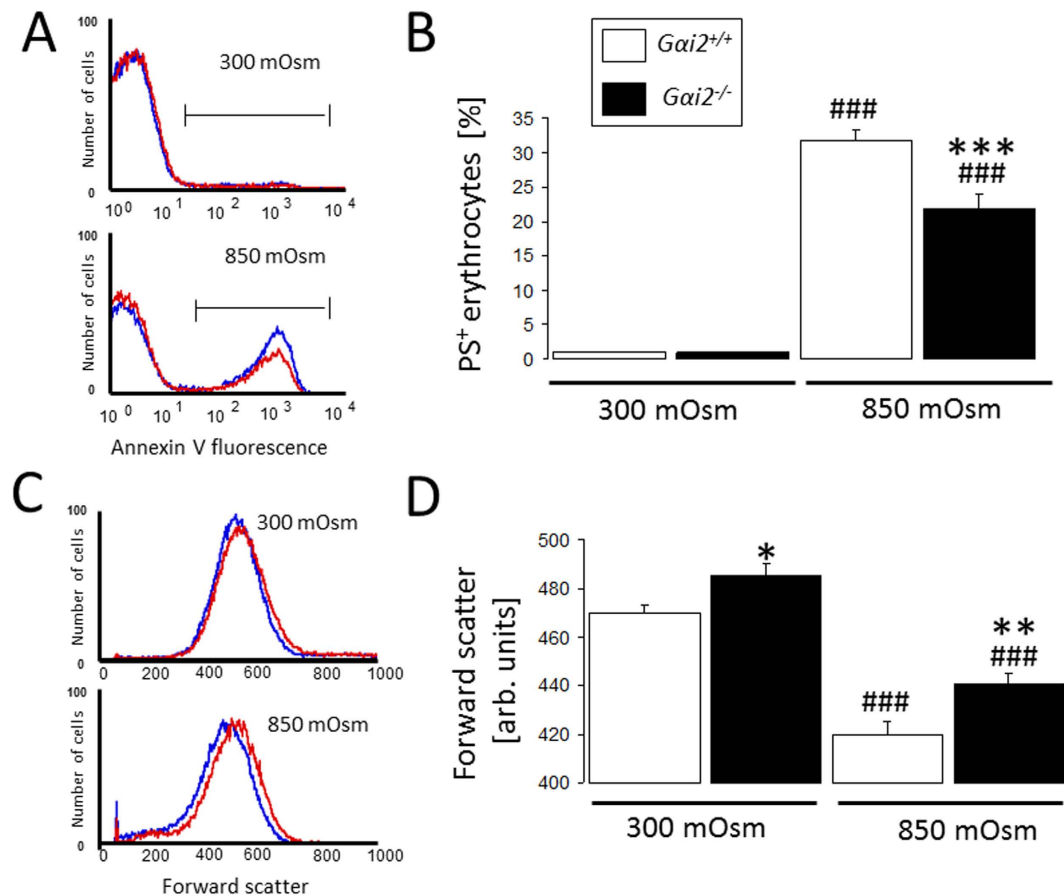


Figure 5. Effect of hyperosmolarity on phosphatidylserine externalization and cell shrinkage. Histogram (A,C; Blue: *Gai2*^{+/+}, red: *Gai2*^{-/-}) and means ± SEM of annexin-V-binding (B, n = 11–14) and forward scatter (D, n = 11–14) following 30 min incubation in isosmotic (300 mOsm) or hyperosmotic (850 mOsm) Ringer. ^{###}(p < 0.001) significantly different from isosmotic, ^{*****}(p < 0.05, p < 0.01, p < 0.001) from *Gai2*^{+/+}.

In conclusion, the G-protein subunit *Gai2* is expressed in human and murine erythrocytes and participates in the regulation of erythrocyte suicide.

Materials and Methods

Mice. Experiments were performed in *Gai2* knockout mice (*Gai2*^{-/-}) and their wild type littermates (*Gai2*^{+/+}) of 6–9 weeks of age. The mice were generated and initially characterized on a SV129 background¹⁸. Mice were backcrossed on a C57BL6 background and kept under specified pathogen-free (SPF) environment in individually ventilated cages (IVC) to prolong life expectancy^{4,59}. 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

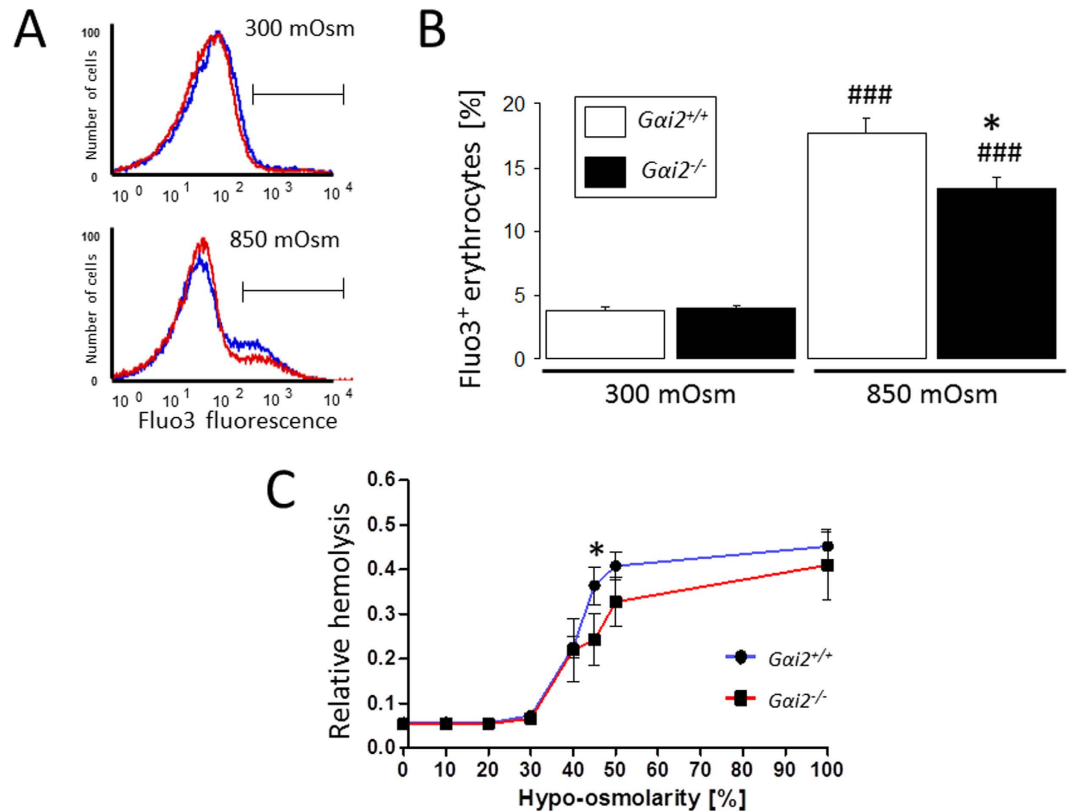


Figure 6. Effect of osmotic changes on cytosolic Ca^{2+} -activity and hemolysis. Histogram (A; Blue: *Gai2*^{+/+}, red: *Gai2*^{-/-}) and means \pm SEM of the percentage of erythrocytes with enhanced Fluo3-fluorescence (B, n = 11–14) following 30 min incubation in isosmotic (300 mOsm) or hyperosmotic (850 mOsm) Ringer. (C) Means \pm SEM (n = 3–5) of relative hemolysis as a function of extracellular osmolarity (in % of isotonic Ringer) in *Gai2*^{+/+} (blue) and *Gai2*^{-/-} (red) erythrocytes. ^{###}(p < 0.001) significantly different from isosmotic, * (p < 0.05) from *Gai2*^{+/+}.

(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 CaCl_2 . The cells were then stained with annexin-V-FITC (1:250 dilution; Immunotools, Friesoythe, Germany) at a 1:500 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 previously²⁴. 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 CaCl_2 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 Ca^{2+} . For measurement of intracellular Ca^{2+} , 50 μl erythrocyte suspension was washed in Ringer solution and then loaded with Fluo-3/AM (Biotrend, Köln, Germany) in Ringer solution containing 5 mM CaCl_2 and 5 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in the fluorescence channel FL-1 in FACS analysis. Where indicated, spontaneous intracellular Ca^{2+} was determined by addition of 2 μl of freshly drawn erythrocytes in 500 μl Ringer solution containing 5 mM CaCl_2 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

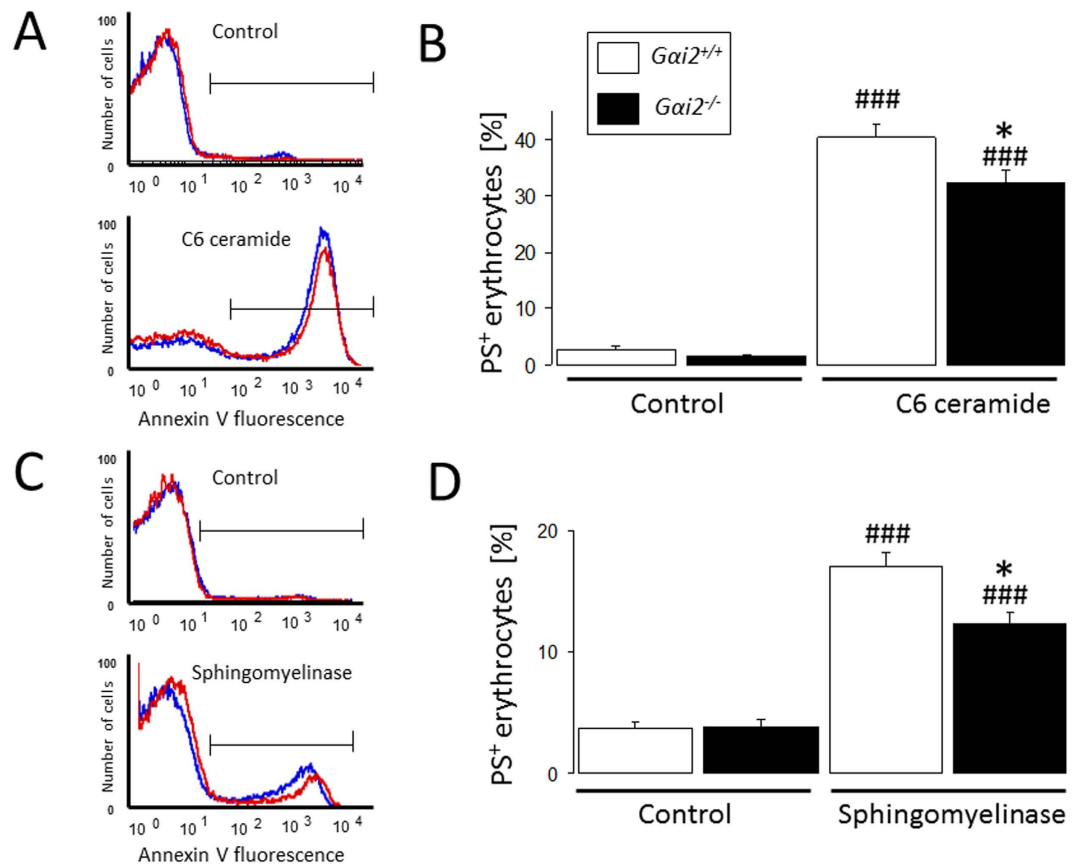


Figure 7. Effect of C6-ceramide and bacterial sphingomyelinase on phosphatidylserine externalization. Histograms (A,C; Blue: *Gai2*^{+/+}, red: *Gai2*^{-/-}) and means \pm SEM of annexin-V-binding following exposure to C6-ceramide (A,B; 50 μ M, 12 h; n = 11–17) or bacterial sphingomyelinase (C,D; 0.01 U/ml 24 h; n = 7–16). ^{###}($p < 0.001$) significantly different from Control. ^{*}($p < 0.05$) from *Gai2*^{+/+}.

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 *Gai2* in human or murine erythrocytes, 150 μ l erythrocyte pellet was lysed in 50 ml 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 μ l 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, Schnellendorf, 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 enzymes⁶⁰. In all cases, 60 μ g 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 μ l of erythrocytes, incubated in the respective experimental solutions, were stained with FITC-conjugated annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 μ l Ringer solution containing 5 mM CaCl₂. Then, the erythrocytes were washed twice and finally resuspended in 50 μ l of Ringer solution containing 5 mM CaCl₂. Twenty μ l 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 \pm SEM, and statistical analysis was made using ANOVA or *t*-test, as appropriate. n denotes the number of different erythrocyte specimens studied.

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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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