Chapter

Red Blood Cells Actively Contribute to Blood Coagulation and Thrombus Formation

Ingolf Bernhardt, Mauro C. Wesseling, Duc Bach Nguyen and Lars Kaestner

Abstract

The chapter describes the likely molecular mechanisms leading to the aggregation of human red blood cells (RBCs) under conditions of physiological coagulation when prostaglandin E_2 (PGE_2) or lysophosphatidic acid (LPA) is released from activated platelets and under pathophysiological conditions, in particular thrombi formation in sickle cell disease when patients are in a vaso-occlusive crisis. In both scenarios cation channels are activated. This leads to an increase of the free intracellular Ca^{2+} concentration resulting in an activation of the lipid scramblase, which in turn mediates a movement of phosphatidylserine (PS) from the inner to the outer membrane leaflet. In addition, the increased Ca^{2+} concentration leads to the activation of the Gardos channel. Experiments suggesting this mechanism have been performed with fluorescence microscopy, flow cytometry as well as single-cell force spectroscopy. The Ca^{2+}-triggered RBC aggregation force has been identified to be close to 100 pN, a value large enough to play a significant role during thrombus formation or in pathological situations.

Keywords: red blood cells, intracellular Ca^{2+} concentration, phosphatidylserine, cation channels, lipid scramblase, thrombus formation

1. Introduction

It is well known that phospholipids are asymmetrically distributed in the cell membrane of most, if not all, living cells. Sphingomyelin (SM) and phosphatidylcholine (PC) are located predominantly in the outer leaflet of the membrane bilayer, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located mostly in the inner leaflet [1] as depicted in **Figure 1A**. **Figure 1B** shows the possible movements of phospholipids from one leaflet of the membrane to the other one (for explanation see text below).

The distribution of the membrane phospholipids is realised by three main proteins: flippase [4], floppase and scramblase [2, 5, 6]. The flippase (also named aminophospholipid translocase (APLT)) transports relatively quickly (in some minutes) PS, and a bit slower PE, from the outer to the inner membrane leaflet. The floppase transports PC and SM in the opposite direction, i.e. from the inner to the outer membrane leaflet [2]. The flippase and floppase are ATP-dependent

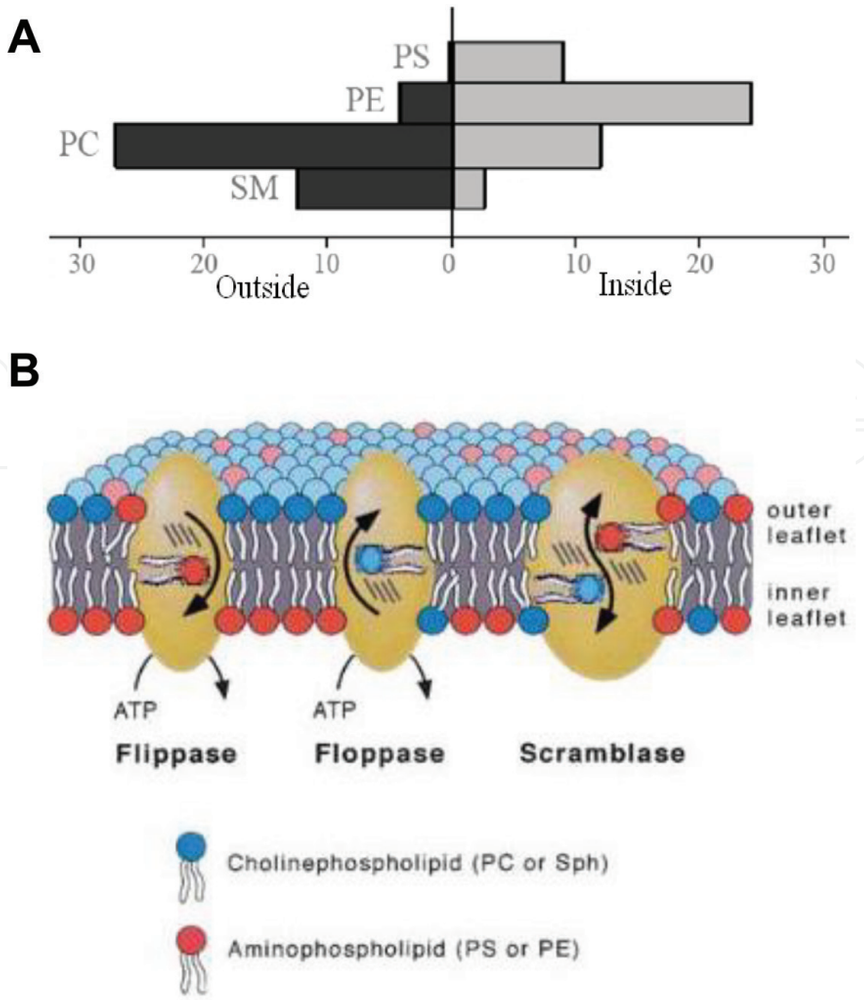

Figure 1.
(A) The asymmetric distribution of phospholipids in the human red cell membrane. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. (B) Transporter-controlled exchange of phospholipids between both lipid leaflets of the cell membrane. Unidirectional phospholipid transport by flippase is directed inwards, whereas floppase promotes outward directed transport. Both transporters are ATP-dependent and frequently move phospholipids against their respective concentration gradients. For example, aminophospholipid translocase (flippase) rapidly shuttles PS and PE from outer to inner leaflet, while ABCC1 (floppase) moves both choline phospholipids and aminophospholipids more slowly towards the outer leaflet. The concerted action of both transporters is thought to create a dynamic asymmetric steady state, in which the outer monolayer is rich in choline phospholipids, whereas aminophospholipids predominantly occupy the inner leaflet. Bidirectional phospholipid transport is catalysed by a scramblase, the activation of which may occur following Ca^{2+} increase. Since scramblase activity moves all major phospholipid classes back and forth between the two leaflets, it promotes the collapse of membrane phospholipid asymmetry with appearance of PS at the cells' outer surface. Panel (A) is modified from [2], and (B) is reproduced from [3].

and transport the phospholipids against their respective concentration gradients. The structure of the flippase has been published recently [7]. The scramblase is ATP-independent and activated by an increasing intracellular Ca^{2+} concentration in human red blood cells (RBCs) [8]. The scramblase has been identified recently as a member of the TMEM16 protein family, and the crystal structure was published [9]. The activity of the three proteins is shown in **Figure 1B**. PS exposure on the outer leaflet of the cell membrane has been described as a marker for apoptosis in nucleated cells [5]. Although the programmed cell death of RBCs is still under discussion, these cells show some signs of apoptosis such as PS exposure, membrane blebbing and vesicle formation [10]. This process was denominated eryptosis by Lang et al. [11]. However, the use of this term is very controversial and finally not recommended [12].

Based on findings showing a correlation between decreased haematocrit and longer bleeding times [13] and experiments of Andrews and Low [14], an active role of RBCs in thrombus formation has been proposed [14]. Kaestner et al. suggested a more detailed signalling cascade based on Ca^{2+} uptake via a non-selective cation channel that could be activated by prostaglandin E_2 (PGE_2) and/or lysophosphatidic acid (LPA) [15–17]. Recent considerations suggest more complicated signalling cascades to be involved [18, 19], including the participation of the mechanosensitive channel Piezo1 [20, 21].

Although it is known that LPA induces PS exposure on the outer membrane leaflet in RBCs, there are conflicting reports about the mechanism. While Chung et al. [22] claim that it is a totally Ca^{2+}-independent process, we showed that Ca^{2+} alone is sufficient to induce PS exposure in human RBCs [23]. Woon et al. found that an increase of the intracellular Ca^{2+} level in RBCs results in the exposure of PS to the outer membrane leaflet due to the activation of the scramblase and inhibition of the flippase [24]. Protein kinase Cα (PKCα) has been also described to be involved in the PS exposure on RBCs [22, 25, 26]. A further discussion follows below.

An interesting model for lipid studies we consider below is sheep RBCs since it is known that these cells have a completely different phospholipid distribution in the membrane. Like in human RBCs, PS and PE are distributed in the inner membrane leaflet. In contrast, the sheep RBC (like all bovine RBC) membrane does not contain any PC, and the outer layer consists exclusively of SM [27, 28].

2. Stimulation of intracellular Ca^{2+} increase in human RBCs

PGE_2 and LPA are local mediators released by platelets after their activation within the coagulation cascade. PGE_2 can be even released by the RBCs themselves under conditions of mechanical stress [29]. We were able to show that the addition of both mediators to suspensions of human RBCs leads to an increase of the intracellular free Ca^{2+} concentration in these cells. In the case of PGE_2 (0.1 nM), 45% of the RBCs responded with increased Ca^{2+} content; in the case of LPA (5 μM), nearly all cells reacted [17, 30] but still showed a strong heterogeneity in the time course and the intensity of the response [31].

The identity of the Ca^{2+} entry was proposed to be channel-mediated; on the one hand, the $Ca_V2.1$-channel [32] and on the other hand the non-selective voltage dependent cation channel [17] were suggested. While the activation of $Ca_V2.1$ was linked to protein kinase C (PKC) activity [33], we could show that $Ca_V2.1$ and PKC belong to different signalling branches [18].

However, in a series of papers utilising fluorescence microscopy and flow cytometry, we could link PKCα activity and Ca^{2+} entry [30, 34].

The above described effect is probably mediated by a specific pathway rather than a non-specific leak since the Ca^{2+} entry shows a clear dose-response relationship towards the LPA concentration [35]. For a recent review about Ca^{2+} channels in human RBCs, see [36].

Another known port for Ca^{2+} entry that is likely to have some pathophysiological relevance is the NMDA-receptor. Among the RBCs, NMDA-receptors were first identified in rat RBCs [37] but later also in human precursor cells as well as in circulating RBCs [38]. NMDA-receptors are cation channels permeable to Ca^{2+} and can be activated by homocysteine and homocysteic acid [39].

To extend on this, **Figure 2** provides an overview of the current knowledge about Ca^{2+} entry and the major cellular consequences in different pathophysiological conditions.

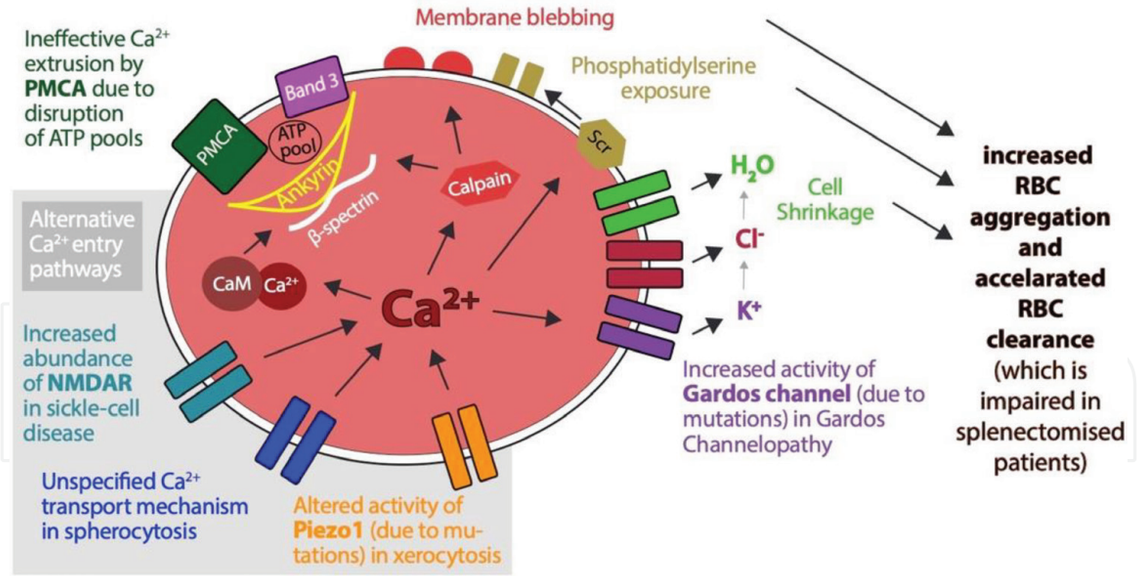

Figure 2.
Proposed mechanisms leading to increased intracellular Ca^{2+} levels in diseased RBCs. Alternative or cumulating Ca^{2+} entry pathways are highlighted with grey background: increased abundance of NMDA-receptors (NMDAR), e.g. in sickle cell disease; altered activity of Piezo1, e.g. in hereditary xerocytosis; increased activity of the Gardos channel, e.g. in Gardos Channelopathy; or unspecified Ca^{2+} transport mechanisms. Additionally, ineffective extrusion of Ca^{2+} due to disruption of ATP pools fuelling the plasma membrane Ca^{2+} ATPase (PMCA) can contribute. Several downstream processes follow Ca^{2+} overload in RBCs, e.g. activation of calmodulin by formation of the Ca^{2+}-calmodulin complex (Ca-CaM) and activation of calpain, thereby loosening the cytoskeletal structure; activation of the scramblase (Scr) leading to exposure of phosphatidylserine on the outer leaflet of the membrane; activation of the Gardos channel followed by the efflux of K^+, Cl^- and H_2O; and consecutive cell shrinkage. All these processes may lead to an increased RBC aggregation and/or accelerated RBC clearance, which is impaired when patients are splenectomised. This figure is modified from Hertz et al. [40].

3. Increased intracellular Ca^{2+} content of human RBCs results in an activation of the scramblase

The PS exposure on the outer membrane leaflet of RBCs was studied for LPA-treated cells using fluorescence microscopy. It has been shown that the number of annexin V-positive cells (i.e. cells showing PS exposure) after LPA treatment in the presence of Ca^{2+} is about 35% [30].

Experiments have also been carried out after treating the RBCs with LPA or the PKC-activator PMA but in the absence of extracellular Ca^{2+} (presence of 1 mM EGTA). In the case of LPA, we did not find significant differences between treatment and control conditions (without activation) regarding PS exposure on human RBCs. Only in the case of PMA treatment, about 50% of RBCs showed PS exposure in the absence of Ca^{2+} (compared to about 80% in the presence of Ca^{2+}). The data obtained for control conditions in the presence of Ca^{2+} were not significantly different from the data of the control conditions in the absence of Ca^{2+} [30].

It is evident that for stimulation with the phorbol ester PMA, there is no correlation between the number of cells showing an elevated Ca^{2+} content and the induced PS exposure, because it is known that PMA activates PKCα also in the absence of Ca^{2+}.

Furthermore we like to state that the threshold for the forward scatter in the flow cytometry measurements was set in a way to make sure that only the events based on the size of intact cells were counted. This was of importance since we realised the formation of micro-vesicles after treatment with LPA as well as with PMA, which is in agreement with findings reported before [41, 42]. Calpain, a Ca^{2+}-dependent

proteolytic enzyme, cleaves spectrin and actin, leading to cytoskeleton breakdown [43–45] and therefore could be involved in the vesiculation process.

The findings that Ca^{2+} content and PS exposure do not correlate in all cells were further supported by confocal fluorescence microscopy, where double-labelling experiments (for intracellular Ca^{2+} and PS in the outer membrane leaflet) have been carried out. The results of the LPA treatment showed that most of the cells have an elevated Ca^{2+} content and depict a PS exposure. However, some cells are displaying an increased Ca^{2+} level, but no PS exposure, whereas other cells externalise PS without having a higher Ca^{2+} content.

In the case of PMA treatment, PS exposure without high Ca^{2+} content is more pronounced, which is not surprising since PMA is an artificial compound, which activates conventional PKCs without the otherwise necessary presence of Ca^{2+}.

The percentage of RBCs showing PS exposure after activation with LPA or PMA is significantly reduced after inhibition of the scramblase using the specific inhibitor R5421 [34]. It is also significantly reduced after the inhibition of the PKCα using chelerythrine chloride or calphostin C. The inhibitory effect is more pronounced when the scramblase and the PKCα are inhibited simultaneously [34].

Based on our experiments, it seems reasonable to assume that at least three different mechanisms could be responsible for the PS exposure on RBCs:

- The first mechanism is related to the Ca^{2+}-stimulated scramblase activation (and flippase inhibition) [4, 8, 10, 11, 47]. This effect involves the activation of the ω-agatoxin-TK-sensitive, $Ca_V2.1$-like (P/Q-type) Ca^{2+} channel [33].

- The second mechanism involves the PKCα, which can be directly activated by PMA. It has been reported that PKCα is involved in the PS exposure in human RBCs [25, 26]. In addition, it has been shown that LPA activates in human RBCs both the PKCα (Ca^{2+}-dependent) and the PKCζ (Ca^{2+}-independent) [22]. Whether there is a direct activation of the scramblase in human RBCs by PKCα and/or PKCζ remains to be proofed. The more pronounced effect of PS exposure observed after treatment of RBCs with PMA as compared to LPA can be explained by assuming that PMA activates all available PKCα, whereas LPA stimulation triggers a signalling cascade [46] resulting only in partial activation of the PKCα pool.

- The third mechanism is the enhanced lipid flop caused by LPA [30]. This mechanism is the only one in sheep RBCs suggesting the absence of scramblase activity in these cells [30].

To consider a possible effect of cell volume alteration on PS exposure, experiments have been carried out where the physiological solution was replaced by a solution containing 150 mM KCl plus 2.5 mM NaCl instead of 145 mM NaCl plus 7.5 mM KCl. Under these conditions of a high extracellular KCl concentration, an opening of the Ca^{2+}-dependent K^+ channel (Gardos channel) does not result in a KCl loss and accompanying cell shrinkage. Based on this strategy, it has been found that a substantial part of the PS exposure in the presence of LPA is caused by the volume decrease.

It remains to be elucidated why, in the experiments reported above, some cells are displaying an increased Ca^{2+} level, but no PS exposure, whereas other cells externalise PS without having a higher Ca^{2+} content. In that context, we carried out single-labelling experiments (fluo-4 for intracellular Ca^{2+} and annexin V-FITC for exposed PS) and focussed on the shape of RBCs using also a fluorescence microscope. The obtained results are presented in **Figure 3**.

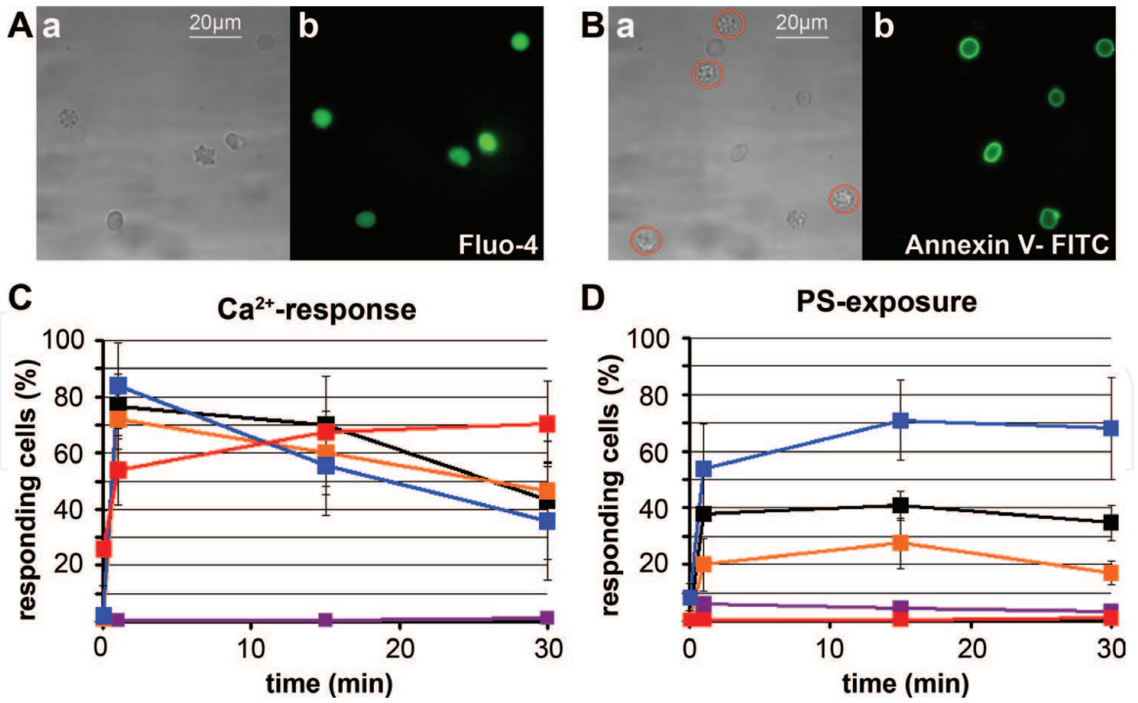

Figure 3.
Human RBC shapes after 2.5 µM LPA stimulation. Transmitted light (left) and fluorescence images (right) of RBCs. (A) Intracellular Ca^{2+} content detected using fluo-4, (B) PS exposure detecting using annexin V-FITC. (C, D) Flow cytometric analysis of responding cells after LPA stimulation in different solutions correlate with predominant cell shapes. Predominantly discocytes, physiological solution (black); predominantly echinocytes, low ionic strength solution + DIDS (red) and physiological solution + DIDS (orange); predominantly stomatocytes or discocytes, low ionic strength solution (blue); physiological solution at pH 5.6 (violet).

In **Figure 3Aa** one can see different cell shapes in the transmitted light image for LPA stimulation. However, the corresponding fluorescence image (**Figure 3Ab**) does not show differences of the increased Ca^{2+} content comparing RBCs with different shapes. The situation changes when analysing the PS exposure of the RBCs with different shapes (**Figure 3B**). Again, RBCs with different shapes can be seen (**Figure 3Ba**), but in the case of PS exposure, the cell shape seems to play a significant role. Some echinocytes can be seen in the transmitted light images (red circles in **Figure 3Ba**). Almost all of them lack PS exposure (corresponding fluorescence image in **Figure 3Bb**). Only one echinocyte showing PS exposure can be observed. The explanation of such an effect could be based on the bilayer couple hypothesis. If an echinocyte is formed, the outer membrane leaflet is already extended compared with the inner membrane leaflet. It means that any process of externalisation of a membrane lipid located mainly in the inner membrane leaflet is hindered. In addition we carried out experiments where we transferred the RBCs to different solutions as shown in
Figure 3C and D. The RBC shapes, which predominantly occur in these solutions, are also indicated in **Figure 3**. Again, the PS exposure is lowest in solution where one can expect mostly echinocytes although the Ca^{2+} content is increased (red curves in **Figure 3**). The PS exposure of RBCs can be small also in solution where cells predominantly occur with the shape of discocytes or stomatocytes, however, in this case only in solutions where the increase of the intracellular Ca^{2+} content is prevented, e.g. physiological solution of low pH (5.6, see violet curves in **Figure 3**).

Furthermore, we carried out double-labelling experiments (X-Rhod-1 for intracellular Ca^{2+} and annexin V-FITC for exposed PS) and investigated the RBCs using a fluorescence microscope. The obtained results are presented in **Figure 4**. One RBC (indicated with white arrow in all panels of **Figure 4**) shows an increased intracellular Ca^{2+} content, but the cell suddenly disappeared from the transmitted light image

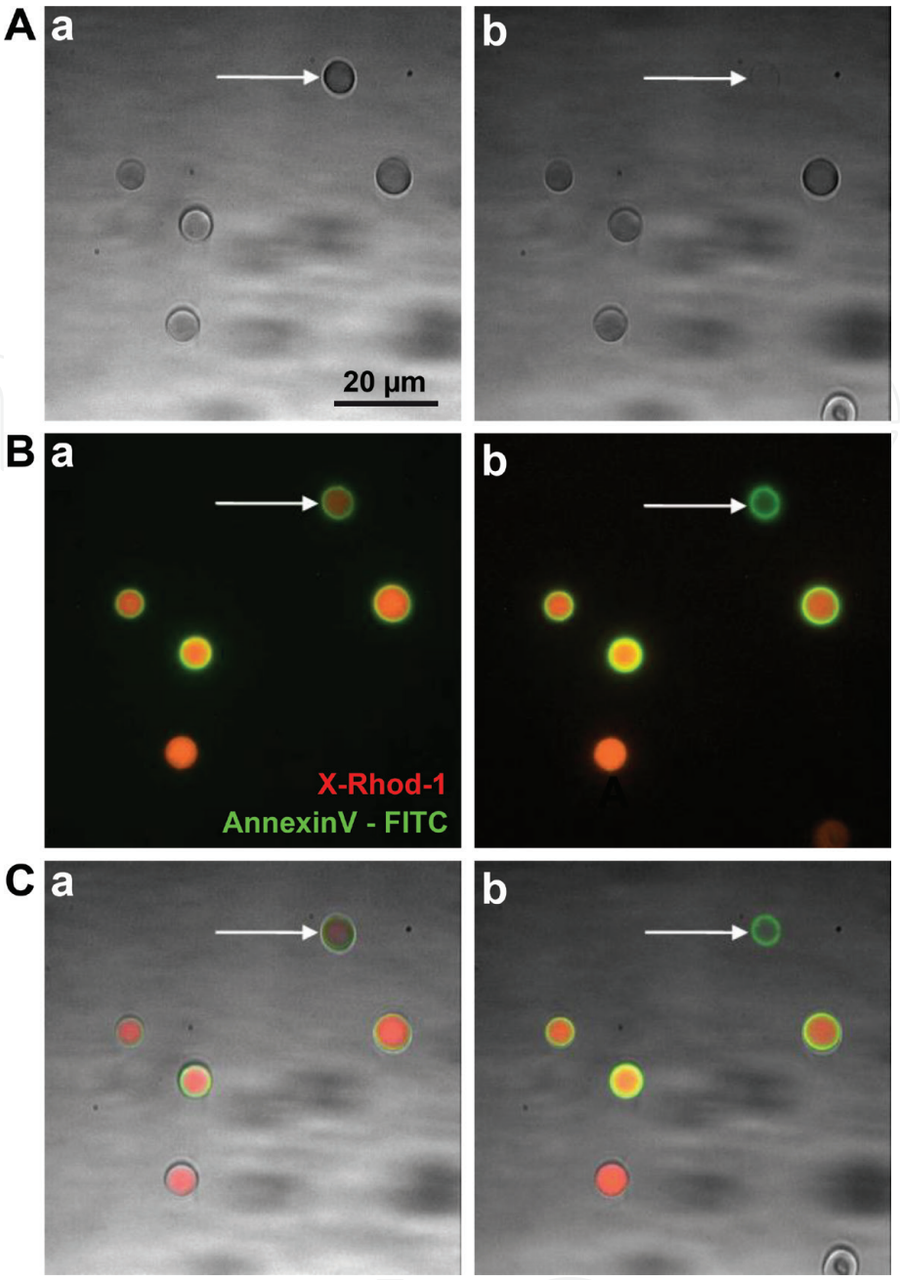

Figure 4.
RBCs were stimulated with lysophosphatidic acid, and panels (A) depict images 1 min after the start of the stimulation and panels (B) 1.5 min after the beginning of the stimulation. Panel (A) shows wide-field images and panel (B) confocal sections of the fluorescent biomarkers X-Rhod-1 (a Ca^{2+} indicator) and FITC-labelled annexin V (for PS detection). Panel (C) is an overlay of panels (A) and (B). The white arrow points to a cell that lysed after capturing images (a), i.e. in images (b) it's a ghost.

(**Figure 4Ab**). However, PS exposure of this cell still can be seen (**Figure 4Bb**). It can be assumed that this RBC lysed, i.e. we see the remaining membrane structure (a ghost) and the Ca^{2+}-sensitive fluorescent dye diffused out of the cell.

A different behaviour of RBCs depending on the age of the cell has been controversially discussed. Therefore, we performed additional short-time incubation experiments, comparable to the experiments carried out by Nguyen et al. [30], with RBCs separated in five fractions with different cell age according to the method of Lutz et al. [47]. The intracellular Ca^{2+} content and PS exposure at the outer membrane leaflet of human RBCs with different age have been investigated using, e.g. LPA stimulation. Here we present a reanalysis of these already published data [48]. Interestingly, the percentage of RBCs showing increased Ca^{2+} content and PS

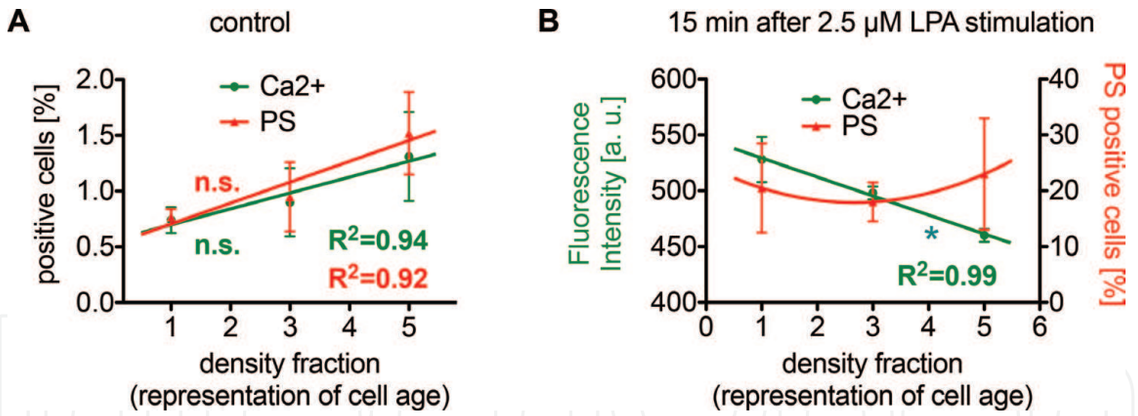

Figure 5.
*Reanalysis of data initially presented in [48]. In the original publication, only two fractions were compared with each other, while here we followed the approach to plot (and analyse) the measured effect in dependence of the cell age. (A) presents the situation under control conditions (without stimulation) and (B) the after 15 min stimulation with LPA ($p < 0.5$ is marked with *).*

exposure depict a linear behaviour in dependence of cell age with a very good regression, R^2 of 0.94 and 0.92, respectively, as outlined in **Figure 5A**. However, the slope of this linear regression failed to be significantly different from zero.

After stimulation of the RBCs with LPA, the situation is even more complex. **Figure 5B** depicts the situation after 15 min of LPA stimulation. While the Ca^{2+} concentration seems to relate inversely proportional to RBC age, PS-positive cells show a rather quadratic dependence on cell age. This is in contradiction to earlier investigations we performed on mouse RBCs [31]. Although we cannot completely resolve the situation part of the explanation might be caused in the detection technique: While microscopy is a rather gentle approach, in flow cytometry the cells under investigation experience high pressure and significant shear forces [60]. Therefore a significant number of high Ca^{2+} cells that are more fragile may lyse in the flow cytometer and hence are excluded from the detection.

Furthermore it is worthwhile to mention that under in vivo conditions, cells with a permanent high Ca^{2+} content and/or PS exposure are removed from the circulation, mostly in the spleen, while after long in vitro incubation time (48 h), old RBCs responded with higher increase of intracellular Ca^{2+} content as well as higher PS exposure compared to young RBCs [48, 49].

The intracellular Ca^{2+} content and the PS exposure at the outer membrane leaflet have been investigated for human RBCs also in physiological solutions prepared with oxygen-enriched water (in comparison to normal physiological solution). This was a study for a company (futomat®) producing equipment for the production of oxygen-enriched water for drinking. It has been found that Ca^{2+} content and the PS exposure are not changed significantly in oxygen-enriched water. However, one interesting effect was found when the RBCs were treated with LPA. Under such conditions the PS exposure was significantly reduced in futomat® water compared to normal physiological solution. It remains open and requires clinical studies to see whether there is a relevant effect in human beings, meaning a positive effect preventing thrombus formation.

4. Active role of RBCs in blood coagulation

When the endothelium of blood vessels is damaged, platelets become activated and transport PS to their external membrane surface [50]. After activation, the exposed PS provides a catalytic surface for the formation of active enzyme-substrate

complexes of the coagulation cascade, especially for the tenase and prothrombinase complexes [51]. Under these circumstances exposed PS provides a procoagulant surface and is, in general, needed as a response to injury. Therefore, the mechanism of PS exposure has to occur with a relative high transport rate of the lipids. Platelets treated with a Ca^{2+} ionophore show a phospholipid scrambling rate of 78×10^3 per second [51]. Human RBCs also show the mechanism of PS exposure after increased intracellular Ca^{2+} content (see above) and are able to adhere to endothelial cells under pathophysiological conditions [22, 52–54]. In addition, exposed PS is sought to serve as a signalling component for macrophages to eliminate old or damaged RBCs from the circulation [55–58]. Since PS-exposing RBCs can adhere to the vascular wall, which may lead to disturbance of the microcirculation [59], the elimination of these cells is a very important mechanism. However, compared to platelets, RBCs have a lower phospholipid scrambling rate (0.45×10^3 per second) [51].

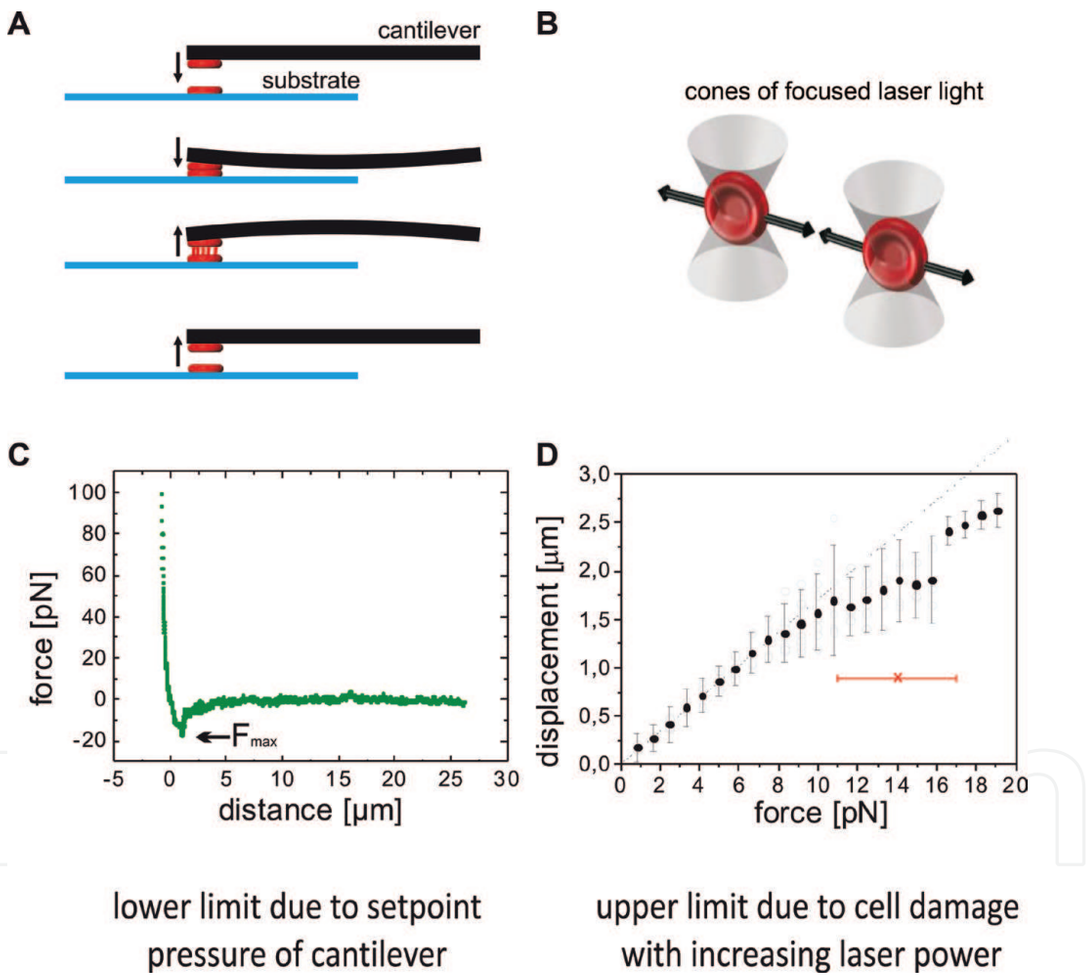

Figure 6.
(A) shows a sketch of the working principle of single-cell force spectroscopy (SCFS). A cell is bound to a cantilever and is brought into contact with another cell at the surface. During the approach and withdrawal of the cell, the deflection is monitored and gives direct information about the adhesion force between the cells. (B) shows a sketch of the working principle of the optical tweezer measurements. Two RBCs are trapped in the foci of two laser beams and are brought into contact. By measuring the deflection of the cells out of the centre of the laser foci, one can determine the adhesion force between the cells. (C) shows a force vs. distance curve derived from the SSFS measurements. A weak interaction of approximately 20 pN can be observed that is mainly due to an artefact of the measurement (see original paper). This 20 pN is the lower limit that one can measure using this type of cell with this technique. (D) shows a force calibration of one RBC in an optical trap. It can be observed that with the given laser power, the trap is only linear up to forces of 15 pN, i.e. this is the upper limit that can be measured with this technique on these types of cells. This figure is reproduced with kind permission from Elsevier. (A) is a reprint from [23], panels (B) and (D) are reprints from [35] and panel (C) is a reprint from [60].

Even more important was the demonstration that human RBCs play an active role in clot formation [23]. This is lacking in medical textbooks, where one can find statements claiming that RBCs only become part of clots because they are so abundant in the circulation. First demonstration of increased interacting forces between two RBCs when the intracellular free Ca^{2+} concentration is increased was performed using non-invasive holographic optical tweezers [23]. In addition, using single-cell force spectroscopy, it has been shown that the upper force limit for Ca^{2+}-triggered adhesion of the RBCs was approximately 100 pN, a value large enough to be of significance during clot formation or in pathological situations [23]. **Figure 6** summarises the in vitro force measurements performed.

5. Active participation of RBCs in thrombotic events

There are numerous indications for the active participation of RBCs in the induction of thrombotic events. The first example we like to mention is the occurrence of thrombotic complications in anaemic patients that experienced a splenectomy. Numerous hereditary anaemic disorders such as spherocytosis, stomatocytosis or elliptocytosis are associated with distorted RBCs, which are preferentially removed in the spleen. Therefore, splenectomy is believed to improve the anaemic symptoms because cells cannot be removed in the spleen. In principle, this concept works out but with the severe side effect that some patients suffer from thrombotic events. Since the 'maintenance' of the RBCs in the spleen is missing, it is likely that the RBCs are the major cause for the thrombotic events. In patients diagnosed with hereditary xerocytosis, mostly related to mutations in the Piezo1 channel, thrombotic complications were regularly reported after these patients underwent splenectomy [61], whereas patients diagnosed for 'Gardos Channelopathy', even after splenectomy, thrombotic events were not observed [62].

An even more prominent example is sickle cell disease associated with vaso-occlusive pain crisis as the major and most severe symptom of the patients. Since the mutation associated with sickle cell disease is in the haemoglobin, it seems obvious that also the symptoms of the disease are associated with RBCs. The common belief is that vaso-occlusive pain crises in sickle cell disease patients are caused by the crystallisation of the mutated haemoglobin under deoxygenation conditions. While the sickle formation under deoxygenation at stasis is undoubted, it's not clear if the same shape change happens in vivo. However, although it is clear that deoxygenated RBCs of sickle cell disease patients have an impaired deformability, the link to the vaso-occlusive crises must be a bit more complicated because deoxygenation happens continuously as deoxygenated RBCs are continuously passing the circulation and vaso-occlusive pain crises happen only sporadically and are so far unpredictable. A possible explanation is the activity of the NMDA-receptors (see above) that are activated by homocysteine and homocysteic acid, which are markers for inflammation in the blood plasma [63].

Such the above described mechanisms triggered by intracellular Ca^{2+} increase are likely to happen also during vaso-occlusive crises in sickle cell disease patients. A first clinical pilot study on sickle cell disease patients using memantine, a drug blocking the NMDA-receptor (and approved to treat Alzheimer disease), showed very promising results both in the support of the mechanism we sketch and in the patients showing a lower number and less severe vaso-occlusive pain crises [64, 65].

Furthermore, it is well known that in RBCs of sickle cell disease patients, the Gardos channel activity is increased [66], which is an indicator for an increased Ca^{2+} since the Gardos channel is a Ca^{2+}-activated K^+ channel. However, a clinical trial testing senicapoc, a Gardos channel inhibitor, failed because vaso-occlusive crises were not improved [66]. Since senicapoc addresses the Gardos channel and

not the upstream increase in Ca^{2+} that causes all the effects described above, the failure of senicapoc is explainable.

Additionally, we like to mention and discuss another aspect: The process we describe, in particular the Ca^{2+}-triggered aggregation to initiate thrombus formation, takes some time [23], and an argument is that the time required is too long that aggregation happens between the fast-moving RBCs in the circulation. In this context, we like to mention the hydrodynamic clustering [67], which is perfectly reversible but provides the cellular interaction time since the lifetime of the hydrodynamic clusters can be in the range of several seconds.

6. Conclusions and outlook

It seems obvious that RBC participation in blood coagulation and thrombus formation is more than an accidental trapping in the process. In this chapter we summarised indications, evidences and proofs for active participation of RBCs in blood clotting and thrombus formation. However, this concept so far did not make it into haematological text books and standard medical education. With this book chapter, we like to make a little contribution to better explain and propagate this concept. Although we face severe experimental and clinical evidence for the active participation of RBCs in blood coagulation and thrombus formation, there is a demand for further research on the regulation and manipulation of this aspect in the coagulation sometimes also referred to as RBC hypercoagulation. We are looking forward to the next years of investigations in coagulation and thrombosis research.

Conflict of interest

The authors don't declare a conflict of interests.

Author details

Ingolf Bernhardt*, Mauro C. Wesseling, Duc Bach Nguyen and Lars Kaestner
Saarland University, Saarbrücken, Germany

*Address all correspondence to: i.bernhardt@mx.uni-saarland.de

References

[1] Haest CWM. Distribution and movement of membrane lipids. In: Bernhardt I, Ellory JC, editors. Red Cell Membrane Transport in Health and Disease. Berlin: Springer; 2003. pp. 1-25

[2] Verkleij AJ, Zwaal RFA, Roelofsen B, Comfurius P, Kastelijn D, Van Deenen LLM. The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. Biochimica et Biophysica Acta. 1973;**323**:178-193

[3] Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. Cellular and Molecular Life Sciences. 2005;**62**:971-988

[4] Devaux PF, Herrmann A, Ohlwein N, Kozlov MM. How lipid flippases can modulate membrane structure. Biochimica et Biophysica Acta. 2008;**1778**:1591-1600

[5] Bevers EM, Comfurius P, Dekkers DW, Zwaal RF. Lipid translocation across the plasma membrane of mammalian cells. Biochimica et Biophysica Acta. 1999;**1439**:317-330

[6] Daleke DL. Regulation of phospholipid asymmetry in the erythrocyte membrane. Current Opinion in Hematology. 2008;**15**:191-195

[7] Perez C, Gerber S, Boilevin J, Bucher M, Darbre T, Aebi M, et al. Structure and mechanism of an active lipid-linked oligosaccharide flippase. Nature. 2015;**542**:433-438

[8] Williamson P, Kulick A, Zachowski A, Schlegel RA, Devaux PF. Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocytes. The Biochemist. 1992;**31**:6355-6360

[9] Brunner JD, Lim NK, Schenck S, Duerst A, Dutzler R. X-ray structure of a calcium-activated TMEM16 lipid scramblase. Nature. 2014;**516**:207-212

[10] Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, Foller M. Eryptosis, a window to systemic disease. Cellular Physiology and Biochemistry. 2008;**22**:373-380

[11] Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. Antioxidants & Redox Signaling. 2006;**8**:1183-1192

[12] Galluzzi L, Vitale I, Aaronson SA, et al. Molecular mechanisms of cell death: Recommendations of the nomenclature committee on cell death. Cell Death and Differentiation. 2018;**25**(3):486-541

[13] Hellem AJ, Borchgrevink CF, Ames SB. The role of red cells in haemostasis: The relation between haematocrit, bleeding time and platelet adhesiveness. British Journal of Haematology. 1961;**7**:42-50

[14] Andrews DA, Low PS. Role of red blood cells in thrombosis. Current Opinion in Hematology. 1999;**6**:76-82

[15] Kaestner L, Christophersen P, Bernhardt I, Bennekou P. The non-selective voltage-activated cation channel in the human red blood cell membrane: Reconciliation between two conflicting reports and further characterisation. Bioelectrochemistry. 2000;**52**:117-125

[16] Kaestner L, Bernhardt I. Ion channels in the human red blood cell membrane: Their further investigation and physiological relevance. Bioelectrochemistry. 2002;**55**:71-74

[17] Kaestner L, Tabellion W, Lipp P, Bernhardt I. Prostaglandin E_2 activates channel-mediated calcium entry in

human erythrocytes: An indication for a blood clot formation supporting process. Thrombosis and Haemostasis. 2004;**92**:1269-1272

[18] Wagner-Britz L, Wang J, Kaestner L, Bernhardt I. Protein kinase Cα and P-type Ca^{2+} channel $Ca_V2.1$ in red blood cell calcium signalling. Cellular Physiology and Biochemistry. 2013;**31**:883-891

[19] Kaestner L. Calcium Signalling: Approaches and Findings in the Heart and Blood. Heidelberg, Berlin, New York: Springer; 2013

[20] Dyrda A, Cytlak U, Ciuraszkiewicz A, Lipinska A, Cueff A, Bouyer G, et al. Local membrane deformations activate Ca^{2+}-dependent K^+ and anionic currents in intact human red blood cells. PLoS One. 2010;**5**:e9447

[21] Kaestner L, Egee S. Commentary: Voltage gating of mechanosensitive PIEZO channels. Frontiers in Physiology. 2018;**9**:1565

[22] Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, et al. Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;**27**:414-421

[23] Steffen P, Jung A, Nguyen DB, Mueller T, Bernhardt I, Kaestner L, et al. Stimulation of human red blood cell leads to Ca^{2+}-mediated intracellular adhesion. Cell Calcium. 2011;**50**:54-61

[24] Woon LA, Holland JW, Kable EP, Roufogalis BD. Ca^{2+} sensitivity of phospholipid scrambling in human red cell ghosts. Cell Calcium. 1999;**25**:313-320

[25] De Jong K, Rettig MP, Low PS, Kuypers FA. Protein kinase C activation induces phosphatidylserine exposure on red blood cells. Biochemistry. 2002;**41**:12562-12567

[26] Klarl BA, Lang PA, Kempe DS, Niemoeller OM, Akel A, Sobiesiak M, et al. Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. American Journal of Physiology. Cell Physiology. 2006;**290**:C244-C253

[27] Nelson GJ. Lipid composition of erythrocytes in various mammalian species. Biochimica et Biophysica Acta. 1967;**144**(2):221-232

[28] Nouri-Sorkhabi MH, Agar NS, Sullivan DR, Gallagher C, Kuchel PW. Phospholipid composition of erythrocyte membranes and plasma of mammalian blood including Australian marsupials; quantitative 31P NMR analysis using detergent. Comparative Biochemistry and Physiology. 1996;**113B**:221-227

[29] Oonishi T, Sakashita K, Ishioka N, Suematsu N, Shio H, Uyesaka N. Production of prostaglandins E_1 and E_2 by adult human red blood cells. Prostaglandins & Other Lipid Mediators. 1998;**56**:89-101

[30] Nguyen DB, Wagner-Britz L, Maia S, Steffen P, Wagner C, Kaestner L, et al. Regulation of phosphatidylserine exposure in red blood cells. Cellular Physiology and Biochemistry. 2011;**28**:847-856

[31] Wang J, Wagner-Britz L, Bogdanova A, Ruppenthal S, Wiesen K, Kaiser E, et al. Morphologically homogeneous red blood cells present a heterogeneous response to hormonal stimulation. PLoS One. 2013;**8**(6):e67697

[32] Yang L, Andrews DA, Low PS. Lysophosphatidic acid opens a Ca(++) channel in human erythrocytes. Blood. 2000;**95**(7):2420-2425

[33] Andrews DA, Yang LPS. Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive

calcium permeability pathway in human red blood cells. Blood. 2002;**100**(9):3392-3399

[34] Wesseling MC, Wagner-Britz L, Nguyen DB, Asanidze S, Mutua J, Mohamed N, et al. Novel insights in the regulation of phosphatidylserine exposure in human red blood cells. Cellular Physiology and Biochemistry. 2016;**39**:1941-1954

[35] Kaestner L, Steffen P, Nguyen DB, Wang J, Wagner-Britz L, Jung A, et al. Lysophosphatidic acid induced red blood cell aggregation in vitro. Bioelectrochemistry. 2012;**87**:89-95

[36] Kaestner L, Bogdanova A, Egee S. Calcium channels and calcium-regulated channels in human red blood cells. In: Islam S, editor. Calcium Signalling. Berlin, Heidelberg, New York: Springer; 2019. In press

[37] Makhro A, Wang J, Vogel J, Boldyrev AA, Gassmann M, Kaestner L, et al. Functional NMDA receptors in mammalian erythrocytes. American Journal of Physiology-Cell Physiology. 2010;**298**:C1315-C1325

[38] Makhro A, Hanggi P, Goede J, Wang J, Bruggemann A, Gassmann M, et al. N-Methyl D-aspartate (NMDA) receptors in human erythroid precursor cells and in circulating red blood cells contribute to the intracellular calcium regulation. American Journal of Physiology-Cell Physiology. 2013;**305**:C1123-C1138

[39] Sibarov DA, Giniatullin R, Antonov SM. High sensitivity of cerebellar neurons to homocysteine is determined by expression of GluN2C and GluN2D subunits of NMDA receptors. Biochemical and Biophysical Research Communications. 2018;**506**:648-652

[40] Hertz L, Huisjes R, Llaudet-Planas E, Petkova-Kirova P, Makhro A, Danielczok J, et al. Is increased intracellular calcium in red blood cells a common component in the molecular mechanism causing anemia? Frontiers in Physiology. 2017;**8**:673

[41] Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, et al. Mechanisms of suicidal erythrocyte death. Cellular Physiology and Biochemistry. 2005;**15**:195-202

[42] Foeller M, Huber SM, Lang F. Erythrocyte programmed cell death. IUBMB Life. 2008;**60**:661-668

[43] Anderson DR, Davis JL, Carraway KL. Calcium-promoted changes of the human erythrocyte membrane. Involvement of spectrin, transglutaminase, and a membrane-bound protease. The Journal of Biological Chemistry. 1977;**252**:6617-6623

[44] Hatanaka M, Yoshimura N, Murakami T, Kannagi R, Murachi T. Evidence for membrane-associated calpain I in human erythrocytes. Detection by an immunoelectrophoretic blotting method using monospecific antibody. The Biochemist. 1984;**23**:3272-3276

[45] Lofvenberg L, Backman L. Calpain-induced proteolysis of beta-spectrins. FEBS Letters. 1999;**443**:89-92

[46] Choi JW, Herr DR, Noguchi K, Yung YC, Lee C, Mutoh T, et al. LPA receptors: Subtypes and biological actions. Annual Review of Pharmacology and Toxicology. 2010;**50**:157-186

[47] Lutz HU, Stammler P, Fasler S, Ingold M, Fehr J. Density separation of human red blood cells on self-forming Percoll gradients: Correlation with age. Biochimica et Biophysica Acta. 1992;**1116**:1-10

[48] Wesseling MC, Wagner-Britz L, Huppert H, Hanf B, Hertz L, Nguyen

DB, et al. Phosphatidylserine exposure in human red blood cells depending on cell age. Cellular Physiology and Biochemistry. 2016;**38**:1376-1390

[49] Ghashghaeinia M, Cluitmans JC, Akel A, Dreischer D, Toulany M, Köberle M, et al. The impact of erythrocyte age on eryptosis. British Journal of Haematology. 2012;**157**:606-614

[50] Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. Thrombosis and Haemostasis. 2002;**88**:186-193

[51] Williamson P, Christie A, Kohlin T, Schlegel RA, Comfurius P, Harmsma M, et al. Phospholipid scramblase activation pathways in lymphocytes. The Biochemist. 2001;**40**:8065-8072

[52] Betal SG, Setty YB. Phosphatidylserine-positive erythrocytes bind to immobilized and soluble thrombospndin-1 via its heparin binding domain. Translational Research. 2008;**152**:165-177

[53] Closse C, Dachary-Progent J, Boisseau MR. Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. British Journal of Haematology. 1999;**107**:300-302

[54] Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, et al. Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. Blood. 2003;**101**:4625-4627

[55] Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**:3077-3081

[56] Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature. 2000;**405**:85-90

[57] McEvoy L, Williamson P, Schlegel RA. Membrane phospholipid asymmetry as a determinant of erythrocyte recognition by macrophages. Proceedings of the National Academy of Sciences of the United States of America. 1986;**83**:3311-3315

[58] Schroit AJ, Madsen JW, Tanaka Y. *In vivo* recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. The Journal of Biological Chemistry. 1985;**260**:5131-5138

[59] Bouguerra G, Aljanadi O, Bissinger R, Abbès S, Lang F. Embelin-induced phosphatidylserine translocation in the erythrocyte cell membrane. Cellular Physiology and Biochemistry. 2015;**37**:1629-1640

[60] Minetti G, Egee S, Mörsdorf D, Steffen P, Makhro A, Achilli C, et al. Red cell investigations: Art and artefacts. Blood Reviews. 2013;**27**:91-101

[61] Stewart GW, Amess JA, Eber SW, Kingswood C, Lane PA, Smith BD, et al. Thrombo-embolic disease after splenectomy for hereditary stomatocytosis. British Journal of Haematology. 1996;**93**:303-310

[62] Fermo E, Bogdanova AY, Petkova-Kirova P, Zaninoni A, Marcello AP, Makhro A, et al. 'Gardos Channelopathy': A variant of hereditary stomatocytosis with complex molecular regulation. Scientific Reports. 2017;**7**:1744

[63] Wu JT. Circulating homocysteine is an inflammation marker and a risk factor of life-threatening inflammatory diseases. Journal of Biomedical and Laboratory Sciences. 2007;**19**(4):107-111

[64] Hegemann I, Sasselli C, Valeri F, Makhro A, Müller R, Bogdanova A, et al. Memantine Treatment is Well Tolerated by Sickle Cell Patients and Improves Erythrocyte Stability: Phase II Study MemSID; 2019

[65] Bogdanova A, Makhro A, Hegemann I, Seiler E, Bogdanov N, Simionato G, Kaestner L, et al. Improved Maturation and Increased Stability of Red Blood Cells of Sickle Cell Patients on Memantine Treatment; 2019

[66] Ataga KI, Reid M, Ballas SK, Yasin Z, Bigelow C, James LS, et al. Improvements in haemolysis and indicators of erythrocyte survival do not correlate with acute vaso-occlusive crises in patients with sickle cell disease: A phase III randomized, placebo-controlled, double-blind study of the Gardos channel blocker senicapoc (ICA-17043). British Journal of Haematology. 2011;**153**:92-104

[67] Brust M, Aouane O, Thiébaud M, Flormann D, Verdier C, Kaestner L, et al. The plasma protein fibrinogen stabilizes clusters of red blood cells in microcapillary flows. Scientific Reports. 2014;**4**:4348