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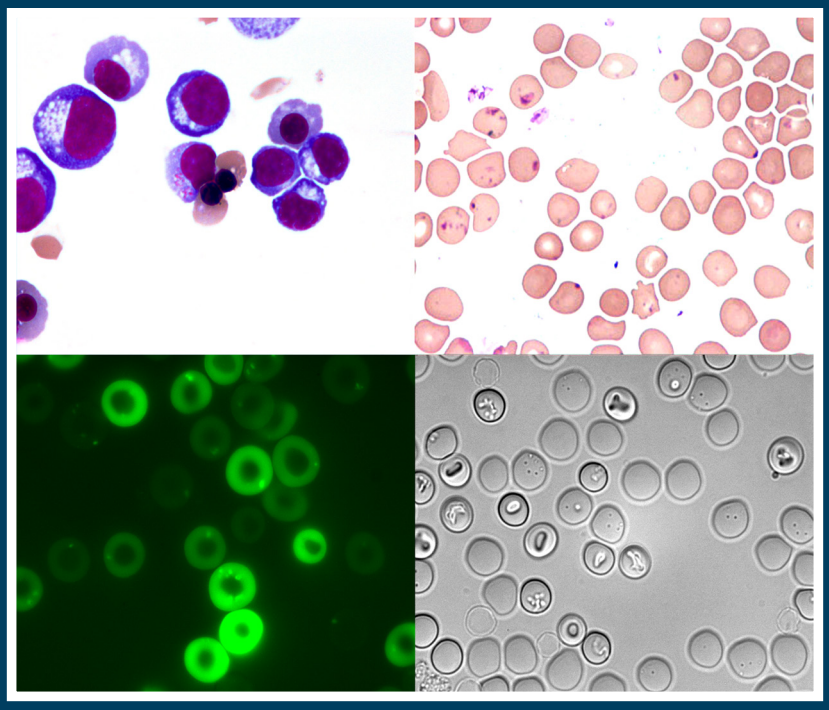

REGULATION OF RED CELL LIFE-SPAN, ERYTHROPOIESIS, SENESCENCE AND CLEARANCE

Topic Editors
Lars Kaestner and Anna Bogdanova

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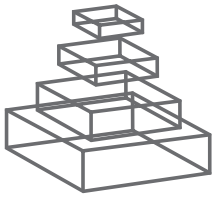

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REGULATION OF RED CELL LIFE-SPAN, ERYTHROPOIESIS, SENESCENCE AND CLEARANCE

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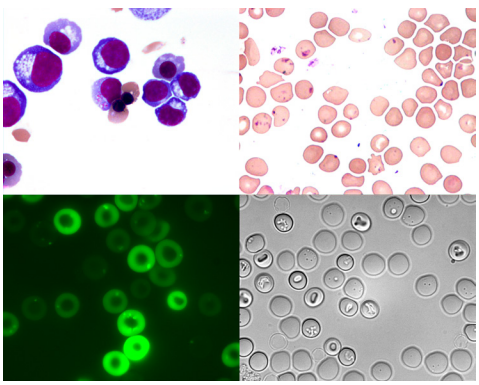

Upper left panel: Ex vivo erythropoiesis. Erythroid precursor cells of sickle cell disease patient at various stages of differentiation in culture (Cytospin). Upper right panel: Reticulocytes of a healthy donor in culture (Cytospin). Lower left panel: Calcium in red blood cells of a sickle cell disease patient visualized by fluo-4. Intracellular vesicles filled with Ca^{2+} are visible Lower right panel: Bright field image of red blood cells of an elite athlete after excessive NMDA receptor stimulation.
Images are produced by Pascal Hänggi, Asya Makhro and Anna Bogdanova

Human red blood cells are formed mainly in the bone marrow and are believed to have an average life span of approximately 120 days. However, is it true for all red blood cells? What are the changes associated with red cell maturation, adulthood and senescence? What are the determinants of red cell life span and clearance? What are the mechanisms in control of red cell mass in healthy humans and patients with various forms of anemia? What are the markers of circulating red cell senescence and in cells during storage and transfusion?

Within the life span may properties of red cells change leading to age-mixed circulating cell populations. Although these cells appear to be genetically terminated by the time they are released into the blood stream, they undergo surprisingly versatile modifications depending on the life-style and health conditions of a "human host". Numerous disorders are believed to be associated with facilitated ageing of red blood cells. "In vitro ageing" and damage of red blood cells during storage is yet one more important issue related to the risks and efficiency of blood transfusion.

Many of the mechanisms behind such effects are far from being fully understood. In this context the Research Topic is set to include articles in the field of biochemical investigations, biophysical approaches, physiological and clinical studies related to red blood cell maturation and aging. This includes Original Research, Methods, Hypothesis and Theory, Reviews and Perspectives.

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EDITORIAL
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Regulation of red cell life-span, erythropoiesis, senescence, and clearance

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Keywords: erythrocyte, erythropoiesis, senescence, clearance, blood storage, neocytolysis, vesiculation

The number of red blood cells (RBCs) and their properties are optimized by nature for most efficient oxygen delivery from the lungs to hypoxic periphery. Changes in metabolic requirements or environmental oxygen availability quickly translate to modulation of the RBC number, blood rheology, oxygen affinity of hemoglobin, and even of vascular tone. Inability to match the changes in oxygen demand may be fatal and requires therapeutic intervention. The recent advances in the ongoing intensive investigations of the mechanisms in control of regulation of erythropoiesis, RBC maturation and aging, as well as the processes involved in recognition of senescent RBCs and their clearance make up the present volume.

It all starts from within the mesoderm, the fetal liver and from the adult bone marrow where primitive or definitive erythropoiesis takes place (Palis, 2014). Facilitated RBC production may be induced promptly "on demand" in extended oxygen requirements upon ascent to the high altitude and quickly reversed when extra RBC mass is without benefit any more (Risso et al., 2014). Exercise and professional sport increase RBC turnover and maximize oxygen delivery to the tissues (Mairbäurl, 2013). Maturation and aging of RBCs is accompanied by multiple processes occurring at various rates driving the circulating RBCs from adolescence to senescence within approximately 120 days (Lew and Tiffert, 2013; Lutz and Bogdanova, 2013). The resulting "markers of senescence" are recognized by the macrophages and clearance of RBCs is promptly initiated (de Back et al., 2014). Premature clearance is a hallmark of various disorders associated with anemia. In each case one or multiple markers of senescence appear prematurely. Those include excessive oxidative stress (Mohanty et al., 2014), excessive cation leak with the following dehydration (Wang et al., 2014), decrease in RBC size and loss of RBC membrane through vesiculation (Alaarg et al., 2013), metabolic abnormalities (Vives-Corrons et al., 2013), or following autoimmune diseases (Lutz and Bogdanova, 2013). Blood storage damages RBCs facilitating aging. As a result clearance of transfused cells is dramatically facilitated (Bosman, 2013; Flatt et al., 2014).

The present compilation does not only give an overview of the variety of opinions reflecting the current understanding of the mechanisms of erythropoiesis, aging, and clearance of RBCs. We hope that it also provides the base for future lively discussions of the up-standing problems in this rapidly developing research area.

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OPINION ARTICLE
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The terminal density reversal phenomenon of aging human red blood cells

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That red blood cells (RBCs) become progressively denser with age has become a universally acknowledged truth. However, as with all universally acknowledged truths, troublesome singularities often arise. We focus here on one particular departure from the rule, the terminal density reversal (TDR) phenomenon, discovered by Bookchin (Bookchin et al., 2000), and documented so far in RBCs from healthy subjects (Bookchin et al., 2000; Lew et al., 2007), from sickle cell anemia patients (SS RBCs) (Bookchin et al., 2000; Franco et al., 2000; Lew et al., 2002; Holtzclaw et al., 2002), from patients with β-thalassemia intermedia (Bookchin et al., 2000), and from diabetic subjects with sustained high levels of glycated hemoglobin, Hb A1c (Bookchin et al., 2009).

There are a number of reasons that justify focusing on TDR: it is of substantial physiological and pathophysiological relevance as it defines the late and terminal homeostatic condition of RBCs in the circulation both in health and disease; it remains controversial and in need of further independent confirmation; its mechanism is still poorly understood, and on the evidence so far it is not yet clear whether it is the common final path of all RBCs or of only a selected RBC subpopulation.

Let us start with a brief review of the facts. TDR was first reported by Bookchin et al. (2000). The original observations were made while studying the time-dependent changes in the volume distribution patterns of K^+-permeabilized RBCs suspended in plasma-like media, as they became progressively dehydrated by the net loss of KCl and water. Bookchin's surprising discovery was that small fractions of cells (0.03 to 4%) failed to dehydrate, both in RBC samples from normal subjects (about 0.05%) and from patients with sickle cell anaemia (about 4%). Because of certain additional clues he speculated that these dehydration-resistant cells could represent a pre-terminal condition of RBCs in the circulation. Scepticism by his co-worker (Virgilio L. Lew), who suspected an artifactual origin of the results, led to the extended investigation that finally confirmed Bookchin's original findings and supported his interpretations. A brief explanation as to why these findings were so surprising follows below.

The two most widely used procedures to selectively increase the K^+ permeability of RBCs in suspension are addition of suitable concentrations of the K^+ ionophore valinomycin or of a divalent cation ionophore, A23187 or ionomycin, in the presence of Ca^{2+} in the medium. Valinomycin acts directly as the K^+ pathway whereas the divalent cation ionophore requires down-gradient Ca^{2+} influx in excess of the powerful Ca^{2+}-extrusion capacity of the plasma membrane calcium pump (PMCA), to build up $[Ca^{2+}]_i$ to activating levels for the endogenous Ca^{2+}-sensitive K^+ channel of the RBC plasma membrane (kcnn4, Gardos channel). Extensive data from many laboratories had shown that when RBCs are suspended in isotonic, low-K media, both these procedures cause rapid dehydration of the cells by the net loss of KCl and water, driven by the outwardly directed electrochemical gradient of K^+, and rate-limited by the anion permeability of the cells.

So the failure of some cells to dehydrate was against all previous experience. After ruling out exotic possibilities such as a low anion permeability or ionophore-resistance, the only explanation left was that dehydration-resistance had to result from unexpected sodium gains balancing potassium loses thus preventing dehydration and densification of the cells in low-K media. But how? Scepticism, rooted on experience, was based on one of the side-effects of ionophore addition, not generally reported. Ionophore incorporation in RBCs membranes always causes haemolysis of a small fraction of cells, intensified when the cells are additionally Ca^{2+}-loaded (Tiffert et al., 1984). It seemed plausible then that small proportions of pre-haemolytic cells had become leaky to cations, gained sodium and thus resisted dehydration during the procedures originally applied by Bookchin.

So the first and most important test was to establish whether the dehydration-resistant cells had gained sodium *in vivo* or *in vitro*. This was resolved in a series of experiments aimed at preventing any possible sodium gains *in vitro* by repeatedly washing RBCs from freshly extracted blood with large volumes of sodium-free isotonic media before testing for dehydration resistance. The sodium and potassium content of untreated RBCs, and of ionophore-treated RBCs, both dehydrated and dehydration-resistant, was measured by atomic absorption spectroscopy in samples from sickle cell anaemia patients and by X-ray microanalysis in single cells from healthy subjects. In all instances, dehydration-resistant cells exhibited reversed Na/K ratios relative to whole blood controls and to ionophore-dehydrated cells, particularly dramatic in RBCs from normal subjects where the Na/K ratio of dehydration-resistant cells was 10 to 18 fold higher than that in the dehydrated cells (Bookchin et al., 2000). These results established that the high sodium content of the dehydration-resistant cells had been gained *in vivo* in the circulation, and that there were real red cell subpopulations whose intravascular condition was of high-Na, low-K, the vast majority of RBCs.

Ancillary measurements by Minetti and collaborators (Minetti et al., 2001) showed that in freshly drawn normal RBCs the increase in sodium content with cell density appeared to be gradual reflecting either progressive net sodium gain with cell age or the redistribution pattern of the high-sodium cells among the density fractions, or both.

The next questions about these dehydration-resistant cells concerned their physiological and pathophysiological significance, and the mechanisms by which they become high-Na, low-K in the circulation. Bookchin's original suspicion that these cells represented a terminal circulatory condition was based on them being found highly enriched among the lightest, reticulocyte-rich fraction of SS RBCs, and with a shape reminiscent of that of the hyperdense irreversible sickled cells (ISCs), as if towards their demise from the circulation they had become sodium-loaded, light and thus dehydration-resistant following K^+-permeabilization. All these initial results were confirmed by additional studies on SS RBCs which also revealed a highly increased pump-leak sodium-potassium traffic across the membrane of the dehydration-resistant cells, with an unexpected bumetanide-sensitive flux-component (Bookchin et al., 2000; Holtzclaw et al., 2002).

In an elegant series of experiments, Franco and collaborators (Franco et al., 2000; Holtzclaw et al., 2002) labelled and re-infused dense SS RBCs into the original donors and followed their appearance among the light cell fractions, unambiguously demonstrating their formation from denser cells. They showed that it was possible to rehydrate dense sickle cells *in vitro* by continuous oxygenation and by rapid oxy/deoxy cycling, that the *in vitro* rehydrated cells were sodium loaded and potassium depleted, that bumetanide inhibited rehydration in oxy conditions, and, most importantly, that the presence of calcium in the medium was an absolute requirement for rehydration.

This calcium dependence opened the possibility of investigating TDR in normal RBCs from healthy subjects, which was much more difficult than in SS RBCs because of their minute proportion. Using the fraction of glycated hemoglobin, Hb A1c, as a reliable measure of RBC age, and newly developed methodologies to separate cells with different calcium pumping capacity (PMCA V_{max}) (Lew et al., 2003, 2007), we were able to define the modality of PMCA V_{max} decline with RBC age and also separate a small fraction of high-Na and high-Hb A1c RBCs, which resisted dehydration when exposed to ionophore A23187 and calcium. These results demonstrated that, as with SS RBCs, the subpopulation of high-Na normal RBCs represented old RBCs near the end of their circulatory life span.

The next question, as to how aging RBCs cells become Na^+-loaded and K^+-depleted *in vivo* by Ca^{2+}-dependent processes, remains open. The high permeability of the dehydration-resistant SS RBCs documented in the early studies suggested an age-dependent activation of a poorly-selective cation permeability pathway, tentatively named Pcat, and challenging suggestions about its possible molecular nature have recently been advanced (Thomas et al., 2011).

The following is presented as a tentative working hypothesis on the mechanism of TDR cell formation based on the available evidence. As RBCs age, PMCA V_{max} declines sharply (Lew et al., 2007). The consequent progressive increase in $[Ca^{2+}]_i$ was estimated to be within the 20 to 100 nM range, too low to be detectable by calcium measurements *ex vivo* (Tiffert and Lew, 1997), yet sufficient to increase the time-averaged open-state probability of the Gardos channels (Grygorczyk, 1987), leading to a sustained net loss of KCl and water, with consequent cell densification, the dominant trend for most of the RBC life span. At some stage along this process, near the end of the cell's circulatory life, calpain proteolysis stimulated by the sustained high $[Ca^{2+}]_i$ levels, or increased membrane protein phosphorylation resulting from macromolecular crowding within the dense cells (Ciana et al., 2004), or some other mechanism yet to be identified, trigger Pcat activation initiating net NaCl gain. This reduces the dehydration rate led by net KCl loss and prevents RBC densification to levels incompatible with normal circulatory flow thus extending the viable RBC life span. Eventually, as the K^+ gradient dissipates, the homeostatic balance progresses to a stage in which net NaCl gain exceeds net KCl loss beyond the restorative capacity of the sodium pump, causing cell rehydration and terminal density reversal in some or all RBCs.

All the evidence discussed thus far points to TDR as a real phenomenon. However, Lutz and collaborators (Alaia et al., 2009) provided data hard to reconcile with TDR being the general terminal homeostatic path of all RBCs. Using a direct antiglobulin test (DAT) for IgG, they examined density fractionated normal RBCs to detect those with elevated numbers of IgG molecules on their surface. Bound IgG not only contained anti-band 3, but also anti-idiotypic and anti-C3 naturally occurring antibodies. The latter two suppress complement-dependent RBC phagocytosis and were thus expected to prolong RBC life span. They found that IgG-DAT-positive blood donors carried most IgG molecules on dense RBCs and had more RBCs of high density than DAT-negative controls. Their densest RBCs were older than the oldest RBCs of DAT-negative controls, based on the band 4.1a/b ratio, an alternative RBC age measure. They argued that if re-swelling leading to TDR had occurred *in vivo* to a significant extent, it would be hard to explain the presence of the overaged RBCs in the high density fractions documented in their experiments. They speculated that TDR may be the result of mechanical-oxidative damage, known to occur in sickle cells, and also during RBC preparatory washing procedures. But perhaps the critical words here are "significant extent," and TDR is the final path of only a subpopulation of RBCs, hard to detect among other dominant density subpopulations.

In conclusion, although the evidence for the *in vivo* presence of aged, high-Na, low-density RBCs and SS RBCs seems unassailable, TDR remains controversial and in need of further study.

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Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells

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During exercise the cardiovascular system has to warrant substrate supply to working muscle. The main function of red blood cells in exercise is the transport of O_2 from the lungs to the tissues and the delivery of metabolically produced CO_2 to the lungs for expiration. Hemoglobin also contributes to the blood's buffering capacity, and ATP and NO release from red blood cells contributes to vasodilation and improved blood flow to working muscle. These functions require adequate amounts of red blood cells in circulation. Trained athletes, particularly in endurance sports, have a decreased hematocrit, which is sometimes called "sports anemia." This is not anemia in a clinical sense, because athletes have in fact an increased total mass of red blood cells and hemoglobin in circulation relative to sedentary individuals. The slight decrease in hematocrit by training is brought about by an increased plasma volume (PV). The mechanisms that increase total red blood cell mass by training are not understood fully. Despite stimulated erythropoiesis, exercise can decrease the red blood cell mass by intravascular hemolysis mainly of senescent red blood cells, which is caused by mechanical rupture when red blood cells pass through capillaries in contracting muscles, and by compression of red cells e.g., in foot soles during running or in hand palms in weightlifters. Together, these adjustments cause a decrease in the average age of the population of circulating red blood cells in trained athletes. These younger red cells are characterized by improved oxygen release and deformability, both of which also improve tissue oxygen supply during exercise.

Keywords: Hb-O_2 affinity, blood gasses, 2,3-DPG, erythropoiesis, hypoxia inducible factor, ATP, NO, intravascular hemolysis

INTRODUCTION

The primary role of red blood cells is the transport of respiratory gasses. In the lung, oxygen (O_2) diffuses across the alveolar barrier from inspired air into blood, where the majority is bound by hemoglobin (Hb) to form oxy-Hb, a process called oxygenation. Hb is contained in the red blood cells, which, being circulated by the cardiovascular system, deliver O_2 to the periphery where it is released from its Hb-bond (deoxygenation) and diffuses into the cells. While passing peripheral capillaries, carbon dioxide (CO_2) produced by the cells reaches the red blood cells, where carbonic anhydrase (CA) in tissues and red blood cells converts a large portion of CO_2 into bicarbonate (HCO_3^-). CO_2 is also bound by Hb, preferentially by deoxygenated Hb forming carboxy-bonds. Both forms of CO_2 are delivered to the lung, where CA converts HCO_3^- back into CO_2. CO_2 is also released from its bond to Hb and diffuses across the alveolar wall to be expired.

The biological significance of O_2 transport by Hb is well-illustrated by anemia where decreased Hb also decreases exercise performance despite a compensatory increase in cardiac output (Ledingham, 1977; Carroll, 2007), and by improved aerobic performance upon increasing total Hb (Berglund and Hemmingson, 1987). The O_2 dissociation curves in **Figure 1** indicate the advantage of normal vs. anemic Hb showing that the O_2 content in blood varies with the Hb concentration in blood at any given O_2 partial pressure (PO_2). Not only its amount but also the functional properties of Hb affect performance. This is illustrated by the observation that an increased Hb-O_2 affinity favors O_2 loading in the lung and survival in an hypoxic environment (Eaton et al., 1974; Hebbel et al., 1978), whereas a decreased Hb-O_2 affinity favors the release of O_2 from the Hb molecule in support of oxidative phosphorylation when the ATP demand is high, such as in exercising skeletal muscle (for a recent review see Mairbäurl and Weber, 2012).

Despite O_2 transport, red blood cells fulfill a variety of other functions, all of which also may improve exercise performance. Likely the most important one is the contribution of red blood cells in buffering changes in blood pH by transport of CO_2 and by binding of H^+ to hemoglobin. Red blood cells also take up metabolites such as lactate that is released from skeletal muscle cells during high intensity exercise. Uptake into red blood cells decreases the plasma concentration of metabolites. Finally, red blood cells seem to be able to decrease peripheral vascular resistance by releasing the vasodilator NO (Stamler et al., 1997) and by releasing ATP which stimulates endothelial NO formation causing arteriolar vasodilation and augments local blood flow (Gonzalez-Alonso et al., 2002).

This review summarizes the mechanisms by which red blood cells warrant O_2 supply to the tissues with special emphasis on O_2 transport to exercising muscle.

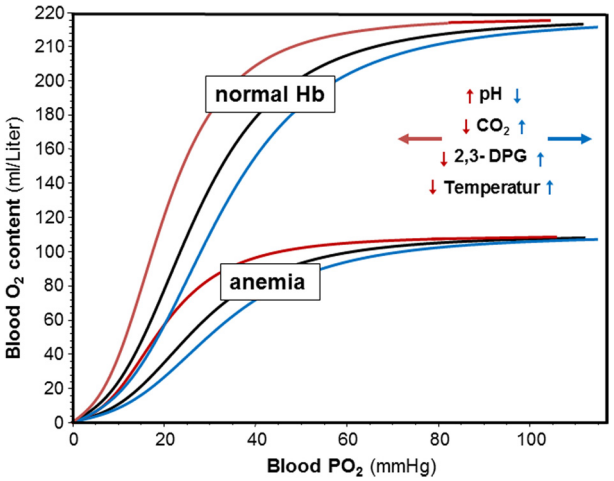

FIGURE 1 | Effects of hemoglobin concentration and pH, CO_2, 2,3-DPG and temperature on blood oxygen content and on Hb-O_2 affinity. Oxygen dissociation curves (ODC) were calculated with the equation by Severinghaus (1979) using decreased, normal, and increased P_{50} values. Oxygen content was calculated from SO_2 and normal and decreased hemoglobin concentrations assuming that 1 g H binds 1.34 ml of O_2. The insert indicates that an increase in pH, and a decrease in CO_2, 2,3-DPG and temperature shifts the ODC to the left (red arrows and curves), whereas acidosis and increased CO_2, 2,3-DPG and temperature shift the ODCs to the right.

OXYGEN AFFINITY OF HEMOGLOBIN

A major mechanism optimizing O_2 transport by hemoglobin is the change in Hb-O_2 affinity. Changes are very fast and actually occur while red blood cells pass through blood capillaries. Effects of altered Hb-O_2 affinity on O_2 transport are independent of Hb concentration and total Hb mass in circulation and thus add to the adjustment by changes in erythropoiesis.

The intrinsic O_2-affinity of hemoglobin is very high (Weber and Fago, 2004). Therefore, allosteric effectors are required that decrease Hb-O_2 affinity allowing unloading of O_2 from the Hb molecule. The major allosteric effectors modulating Hb-O_2 affinity *in vivo* in human red blood cells are organic phosphates such as 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP), H^+ and CO_2, and Cl^-. A direct role of lactate, which accumulates during exercise, on Hb-O_2-affinity is less clear and may be due to a small effect on the Cl^- binding by Hb and on carbamate formation (reviewed in (Mairbäurl and Weber, 2012)). Indirect effects of lactate may be caused by affecting the Cl^- concentration and by the uptake of H^+ together with lactate mediated by MCT-1 (Deuticke, 1982). Another modulator of Hb-O_2 affinity relevant to exercise is a change in body temperature (Dill and Forbes, 1941; Mairbäurl and Humpeler, 1980). **Figure 1** shows that at any Hb concentration, acidosis, and an increase in CO_2 and 2,3-DPG decrease Hb-O_2 affinity. Changes in Cl^- are small *in vivo* and are therefore not shown on the graph. Also an increase in temperature decreases Hb-O_2 affinity. These changes shift the ODC to the right showing graphically that the O_2 saturation of Hb (SO_2) is decreased at any given PO_2. In contrast, alkalosis, a decrease in CO_2, 2,3-DPG, and temperature increase Hb-O_2 affinity to increase SO_2 at a given PO_2.

The physiological significance of an increased Hb-O_2 affinity is an improved O_2 binding by Hb when the PO_2 is low. It is therefore of significance for people exposed to hypoxic environments, where it prevents exaggerated arterial desaturation. A decrease in Hb-O_2 affinity improves O_2 delivery to cells with a high O_2 demand such as in exercising muscle (see below).

A simple approach to estimate the SO_2 from PO_2 and vice versa has been published by Severinghaus (1979). The formula was derived from a best fit model of the standard oxygen dissociation curve with an error of SO_2 of 0.26% within the physiological range of PO_2. The standard half saturation pressure of O_2 (P_{50}value) was given as 26.86 mmHg at a plasma pH = 7.4 and 37°C; S is fractional saturation.

$$S = 100 \times (((PO_2^3 + 150 \times PO_2)^{-1} \times 23400) + 1)^{-1} \text{or}$$

$$\ln PO_{2,st} = 0.385 \times \ln (S^{-1} - 1) + 3.32 - (72 \times S)^{-1} - 0.17 \times S^6$$

Based on a model proposed by Roughton and Severinghaus (1973), Okada et al. (1977) published a modification of this formula that allows estimating changes in P_{50} by altered pH, temperature (T; °C), base excess (BE; mEq/Liter), and 2,3-DPG (DPG; molar ration of 2,3-DPG to Hb) with accuracies of P_{50} values and SO_2 of ± 2.5 and ± 5%, respectively.

$$\Delta \log_{50} = 0.48 \times (7.4 - pH_{plasma}) + 0.024 \times (T - 37) + 0.0013 \times BE + 0.135 \times DPG - 0.116,$$

After correction of P_{50} using this equation to obtain $P_{50,actual}$, adjusted PO_2 ($PO_{2,actual}$) values can be calculated (Severinghaus, 1979) as

$$PO_{2,actual} = PO_{2,std} \times \frac{P_{50,actual}}{26.86}$$

Then the "Severinhaus-equation" can be used to calculate S from the new PO_2 to obtain complete ODCs. A more detailed description of the magnitude of changes in Hb-O_2 affinity by allosteric effectors, temperature, and other molecules alone as well as their interactions is reviewed in (Mairbäurl and Weber, 2012).

Hb-O_2 AFFINITY DURING EXERCISE

During exercise the increased demand for oxygen is met by increasing muscle blood flow (Laughlin et al., 2012) and by improved O_2 unloading from Hb achieved by decreasing Hb-O_2 affinity (Mairbäurl, 1994). It is obvious that a decreased Hb-O_2 affinity, if occurring systemically—i.e., in all red blood cells in circulation—will compromise arterial O_2 loading of Hb in the lung. It would thus be advantageous if adjustments in Hb-O_2 affinity occurred locally to serve both functions, oxygenation in the lung and deoxygenation in peripheral blood capillaries. Thus, Hb-O_2 affinity should be low while red blood cells pass through tissues with a high O_2 demand, and should be increased when red blood cells return to the lung. This is in fact what happens because of distinct differences in pH, CO_2 and temperature between the lung and capillaries in working muscles. No changes in 2,3-DPG, one of the major allosteric effectors of Hb-O_2 affinity (Benesch and Benesch, 1967), during exercise tests have been

observed (Mairbäurl et al., 1986), because 2,3-DPG changes are slow and require adjustments of the glycolytic rate in red blood cells. However, elevated 2,3-DPG has been found after training (Böning et al., 1975; Braumann et al., 1982; Mairbäurl et al., 1983; Schmidt et al., 1988). It might be considered beneficial for O_2 unloading during exercise because it increases the effect of acidosis (Bohr effect) on Hb-O_2 affinity (Bauer, 1969). The elevated 2,3-DPG in trained individuals might be a consequence of the stimulated erythropoiesis, which decreases red blood cell age (Mairbäurl et al., 1983). Young red blood cells have an increased metabolic activity (Seamen et al., 1980; Rapoport, 1986), higher 2,3-DPG, and a lower Hb-O2-affibity than senescent red blood cells (Haidas et al., 1971; Mairbäurl et al., 1990).

O_2 unloading to exercising muscle

Exercising muscle cells release H^+, CO_2, and lactate into blood capillaries, and there is also a higher temperature in working muscle than in inactive tissues. Blood entering capillaries of exercising muscles is acutely exposed to these changes, which causes a rapid decrease in Hb-O_2 affinity. P_{50} values of ∼34–48 mmHg can be estimated from changes in blood gasses (provided e.g., in Sun et al., 2000). Temperature increases from 37°C at rest to 41°C during exercise. Because there is a continuous change in blood composition by admixture of metabolites as new blood enters a capillary, P_{50} values are lower at the arterial side of the capillaries than at their venous end (Mairbäurl and Weber, 2012) causing an enormous rightward shift of the ODC within the capillaries that increases unloading of O_2 from Hb considerably (Berlin et al., 2002). This is also demonstrated by the extensive shift to the right of the ODC in capillary blood in exercise conditions relative to rest (**Figure 2**; points D and B, respectively). Trained individuals have a higher Bohr effect at low SO_2 probably due to elevated 2,3-DPG (Böning et al., 1975; Braumann et al., 1982; Mairbäurl et al., 1983), which might cause an even greater increase in the arterio-to-venous O_2 difference.

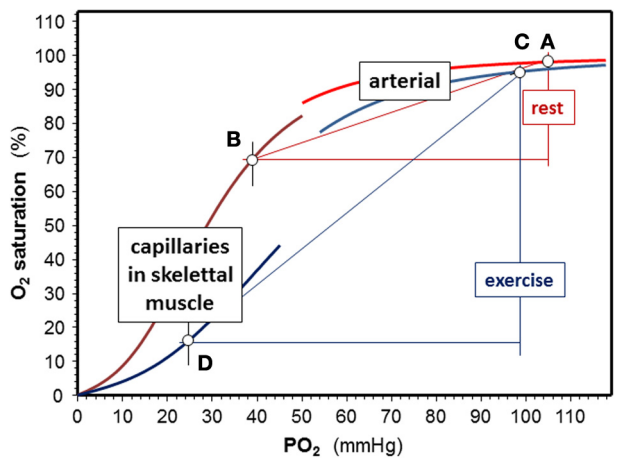

FIGURE 2 | Effects of exercise on Hb-O_2 affinity. Shifts of the O_2-dissociation curves are calculated for an arterial pH = 7.4, a capillary pH = 7.3, and temperature is 37°C rest. Values used to calculate oxygen dissociation curves (ODC) for exercise were an arterial pH = 7.15 at 38.5°C, a capillary pH = 7.0 and temperature = 41°C in working muscle using the equation giving in the text using data from exercise tests (Sun et al., 2000). At rest, assuming a venous PO_2 = 40 mmHg, SO_2 decreases by 28% (points **A** and **B**), while extraction nearly triples in exercise conditions (delta SO_2 = 79%) assuming a venous PO_2 = 25 mmHg during exercise (points **C** and **D**).

Arterial O_2 loading

On its way from working muscle to the lung the concentrations of H^+ and CO_2 in blood are decreased by admixture of blood coming from inactive muscle and other organs. CO_2 decreases in alveolar capillaries due to alveolar gas exchange, which further alkalinizes the blood. Thus, the effects of these metabolites on Hb-O_2 affinity are attenuated in the lung relative to working muscle. Also the temperature is lower in the lung than in working muscle. Nevertheless, normal values of Hb-O_2 affinity are not restored completely during intensive exercise, which is indicated by the slight shift to the right of the ODC in the exercise conditions relative to the resting situation (**Figure 2**; points A and C). The magnitude of the deviation depends on the active muscle mass and exercise intensity. From blood gas data during exercise reported by Wasserman and colleagues (Sun et al., 2000) it can be estimated that the half saturation tension of O_2 (P_{50} value) might increase from about ∼27 mmHg at rest to 34 mmHg in arterial blood during heavy exercise. This decrease in Hb-O_2 affinity impairs arterial O_2 loading and decreases arterial SO_2 from ∼97.5% at rest to ∼95% during high intensity exercise. An increased 2,3-DPG in trained individuals might further decrease arterial SO_2 (Mairbäurl et al., 1983). Adding to the effect of decreased Hb-O_2 affinity, SO_2 decreases further because of diffusion limitation by the shortened contact time when cardiac output is high (Dempsey and Wagner, 1999; Hopkins, 2006), which might even be enhanced when exercise is performed in hypoxia (Calbet et al., 2008).

When comparing the effects of acid metabolites and the increased body temperature during exercise on Hb-O_2 affinity in arterial and muscle capillary blood it is evident that the changes are much greater in working muscle than in the lung. Thus, the greatly increased amount of O_2 unloaded from Hb relative to rest easily compensates for arterial desaturation during exercise.

OXYGEN TRANSPORT CAPACITY

Whereas only 0.03 ml O_2 * L^{-1} * $mmHg^{-1}$ PO_2 at 37°C can be transported in blood in physical solution, one gram of Hb can bind ∼1.34 ml of O_2. Thus, the presence of a normal amount of Hb per volume of blood increases the amount of O_2 that can be transported about 70-fold, which is absolutely essential to meet the normal tissue O_2 demand. It is therefore apparent that an increased amount of Hb also increases the amount of O_2 that can be delivered to the tissues (**Figure 1**). In fact, the O_2 transport capacity was found to correlate directly with aerobic performance as can be seen from an increase in performance after infusion of red blood cells (Berglund and Hemmingson, 1987) and by the strong correlation between total Hb and maximal O_2 uptake ($VO_{2,max}$) in athletes (for review see Sawka et al., 2000; Schmidt and Prommer, 2010). Calbet et al found that acute manipulations of the O_2 carrying capacity also vary performance (Calbet et al., 2006). Thus, it is a clear advantage for aerobic athletic performance to have a high O_2 transport capacity.

Parameters required to evaluate O_2 transport capacity are the Hb concentration in blood (cHb) and hematocrit (Hct), as well as total Hb mass (tHb) and total red blood cell volume (tEV) in circulation. cHb and Hct are easy to measure with standard hematological laboratory equipment. Together with SO_2 they indicate the amount of O_2 that can be delivered to the periphery per unit volume of cardiac output. tHb and tEV indicate the total amount of O_2 that can be transported by blood. A large tHb and tEV allows redirecting O_2 to organs with a high O_2 demand while maintaining basal O_2 supply in less active tissues. Because they are affected by changes in plasma volume (PV) cHb and Hct allow no conclusion on tHb and tEV, respectively.

Results on cHb, Hct and red blood cell count in athletes and their comparison with values obtained in healthy, sedentary individuals are conflicting due to the fact that red blood cell volume and PV change independently and due to the many factors affecting each of these parameters (see below). Establishing normal values for tHb and tEV for athletes is hampered further by the possibility of use of means to increase aerobic capacity such as blood and erythropoietin (EPO) doping.

HEMATOCRIT IN ATHLETES

Many but not all studies show lower Hct in athletes than in sedentary controls (Broun, 1922; Davies and Brewer, 1935; Ernst, 1987; Sawka et al., 2000). However, several studies also report higher than normal Hct. A highly increased Hct increases blood viscosity and increases the workload of the heart (El-Sayed et al., 2005; Böning et al., 2011). It therefore bears the risk of cardiac overload.

Many studies showed that Hct tended to be lower in athletes than in sedentary individuals (Broun, 1922; Davies and Brewer, 1935; Remes, 1979; Magnusson et al., 1984; Selby and Eichner, 1986; Ernst, 1987; Weight et al., 1992). This was verified by Sharpe et al. (2002) in the course of establishing reference Hct and Hb values for athletes. The found that out of ∼1100 athletes from different countries 85% of the female and 22% of the male athletes had Hct values below 44%. A tendency for an inverse correlation of Hct with training status, indicated by $VO_{2,max}$, was also shown (Heinicke et al., 2001). However, a small proportion of sedentary controls and athletes has higher than normal Hct. Sharpe et al. (2002) found that 1.2% of all females and 32% of all males in their study had an Hct > 47%. When following female and male elite athletes and controls over a study period of 43 months Vergouwen (Vergouwen et al., 1999) found 6 males controls and 5 males athletes with a Hct > 50% and 5 females controls but no female athletes with a Hct > 47%.

Hematocrit during exercise

Changes in Hct occur rapidly. Hct increases during exercise due to a decrease in PV when fluid replacement during exercise is insufficient (Costill et al., 1974). There is fluid loss due to sweating, a shift of plasma water into the extracellular space due to the accumulation of osmotically active metabolites, and filtration as a consequence of an increased capillary hydrostatic pressure (Convertino, 1987). The resultant increase in plasma protein increases oncotic pressure and thus moderates fluid escape (Harrison, 1985). Changes appear less pronounced during swimming than running exercise, where immersion and the re-distribution of blood volume seem to cause shifts in PV independent of volume regulatory hormones (Böning et al., 1988). An increase in hematocrit due to catecholamine-induced sequestration of red blood cells from spleen is unlikely in humans but has been found in other species (Stewart and McKenzie, 2002).

Long-term changes of hematocrit

In a recent review, Thirup (2003) reports a within-subject variability of ∼3% when reviewing 12 studies on more than 600 healthy, non-smoking, mostly sedentary individuals, and when measurements were repeated in sampling intervals ranging from days to ∼2 months. Sawka et al. summarized data from 18 investigations and found that PV and blood volume increased rapidly after training sessions, whereas red cell volume remained unchanged for several days before it began to increase indicating that Hct values were decreased for several days (Sawka et al., 2000). The magnitude of Hct change seems to depend on exercise intensity during training sessions and the type of exercise (strength vs. endurance; for review see Hu and Lin, 2012). A few weeks after the training intervention a new steady state had established, and Hct had returned to pre-training values (Sawka et al., 2000). The post-training increase in PV and the increased PV in highly trained athletes (e.g., Hagberg et al., 1998; Sawka et al., 2000; Heinicke et al., 2001; Schumacher et al., 2002) is likely caused by aldosterone dependent renal Na^+ reabsorption, and by water retention stimulated by elevated antidiuretic hormone in compensation for the water loss during individual training sessions (Costill et al., 1976; Milledge et al., 1982).

There appear to be quite large seasonal variations in Hct (relative change up to 15%) with lower values in summer than in winter that might result in season-to-season changes from ∼42% in summer and 48% in winter as found among several thousand study participants. Seasonal changes depend on climatic effects with larger differences in countries closer to the equator (Thirup, 2003). Studies of seasonal changes in Hct of athletes are sparse but indicate that Hct might be decreased by another 1–2% in summer by addition of a training effect.

The decreased Hct in athletes has been termed "sports anemia." For a long time it had been explained with increased red blood cell destruction during exercise and thus appeared to be the same phenomenon as the well-known march hemoglobinuria (Broun, 1922; Kurz, 1948; Martin and Kilian, 1959). Intravascular destruction of red blood cells occurs at shear stresses between 1000 and 4000 dyn/cm^2 (Sutera, 1977; Sallam and Hwang, 1984), values well above physiological values at rest (Mairbäurl et al., 2013). It is related to the intensity and the kind of exercise (Yoshimura et al., 1980; Miller et al., 1988). Foot strike in runners has been the most often reported reason for intravascular hemolysis (Telford et al., 2003), which can be prevented by good shoe cushioning (Yoshimura et al., 1980; Dressendorfer et al., 1992). It also occurred during mountain hiking (Martin et al., 1992), in strength training (Schobersberger et al., 1990), karate (Streeton, 1967), in swimmers (Selby and Eichner, 1986; Robinson et al., 2006), basketball, Kendo-fencing, and in drummers (Schwartz and Flessa, 1973; Nakatsuji et al., 1978). Running exercise has been found to increase plasma hemoglobin from ∼30 mg/liter at

rest to ∼120 mg/liter indicating that about 0.04% of all circulating red blood cells were lyzed (Telford et al., 2003). Exercise had been shown to alter red blood cell membrane appearance in correlation with elevated haptoglobin (Jordan et al., 1998). Senescent red blood cells may be particularly prone to exercise induced intravascular hemolysis as indicated by a decreased mean red blood cell buoyant density and a density distribution curve that was skewed toward younger, less dense cells in trained individuals indicated by increased levels of pyruvate kinase activity, 2,3-DPG and P_{50}, higher reticulocyte counts (Mairbäurl et al., 1983). Other possible reasons for "sports anemia" under discussion are nutritional aspects such as insufficient protein intake and altered profile of blood lipids (for review see Yoshimura et al., 1980), and iron deficiency (Hunding et al., 1981).

TOTAL HEMOGLOBIN MASS (tHb) AND TOTAL RED BLOOD CELL VOLUME (tEV)

As indicated above, PV is prone to acute changes, whereas changes in total red blood cell mass (or volume) are slow due to slow rates of erythropoiesis (Sawka et al., 2000). Therefore, total hemoglobin and/or red blood cell volume has to be measured in addition to cHb and Hct to obtain a reliable measure of the oxygen transport capacity. Several methods have been applied to determine these parameters.

Grehant and Quinquard (1882) were the first to describe blood volume measurements by use of carbon monoxide (CO)-rebreathing. This method is based on the much higher affinity of Hb to CO than to O_2 (for review see Mairbäurl and Weber, 2012), which allows using CO in an indicator dilution method. It has been used to measure the fraction of blood mass relative to body mass by Arnold et al. (1921). The technique has been improved considerably by Sjostrand by advancing the method to estimate carboxy-hemoglobin (Sjostrand, 1948). To date CO rebreathing or inhalation has been further improved (Godin and Shephard, 1972; Schmidt and Prommer, 2005). MCHC is then used to calculate tEV, and Hct to estimate total blood volume. Total red blood cell volume can be determined directly after injection of ^{99m}Tc-labeled red blood cells (Thomsen et al., 1991). By indirect means, total red blood cell volume can also be calculated from Hct after measuring the PV using Evans blue (T-1824), which binds to albumin, and by injection of ^{125}iodine-labeled albumin. Several of these methods have been compared by Thomsen et al. (1991) who reported a correlation of $r = 0.99$ between PV measured by ^{125}I-albumin and Evans blue, and showed that PV calculated from measuring tEV with labeled red blood cells was about 5–10% lower than that from labeling albumin.

Applying these techniques Kjellberg et al. found that trained individuals had increased tHb (Kjellberg et al., 1949), a result that has been confirmed many times thereafter both by comparing groups of individuals with different training status and by measuring tEV before and after prolonged training periods (for a recent review see Sawka et al., 2000). Schmidt and Prommer summarized recently that different training modalities vary in their effects on tHb, where they put the main emphasis on training in hypoxia (Schmidt and Prommer, 2008). In summary, these studies show that an increase in tHb by 1 g achieved e.g., by administration of erythropoietin, increased $VO_{2,max}$ by ∼3 ml/min (Parisotto et al., 2000; Schmidt and Prommer, 2008). From the correlation shown by Heinicke et al. (2001) it can be derived that an increase in 1 g of tHb per kg body weight (g/kg) increased $VO_{2,max}$ by ∼5.8 ml/min/kg, where non-athletes (though with a rather high $VO_{2,max}$ of 45 ml/min/kg) had a tHb of 11 g/kg and their best athletes (average $VO_{2,max} = 71.9$ ml/kg) had a tHb of 14.8 g/kg (Heinicke et al., 2001). Their findings fit well to the results reported by Kjellberg, who found a 37% higher tHb in elite athletes than in untrained individuals (Kjellberg et al., 1949). Schmidt and Prommer (2008) combined results from several of their studies and found a change in $VO_{2,max}$ of 4.2 ml/min/kg in males and of 4.4 ml/min/kg in females per change in tHb of 1 g/kg with very high correlation coefficients ($r \sim 0.79$), whereas there was no correlation between $VO_{2,max}$ and Hb or Hct. However, there are also reports on a lack of difference in tHb between sedentary and trained individuals (Green et al., 1991). As mentioned above all these studies bear the burden of uncertainty that athletes may have taken measures to increase performance, which makes it difficult to establish "normal values" of tHb and tEV for athletes.

Different duration of exercise training (weeks vs. months) appear to explain the diverging results in the studies on tHb and training. Sawka et al. (2000) found no increase when training lasted less than 11 days. Also most studies on 4–12 months of training showed no or only small effects; their own longitudinal study on "leisure sportsmen" resulted in an increase in tHb by ∼6% in the course of a 9-month endurance training (summarized in Schmidt and Prommer, 2008) indicating that adjustments of tHb and tEV by training are slow, and that a pronounced increase may require several years of training.

Sedentary high altitude residents have an increased tHb in comparison to their low altitude counterparts, where blood volume has been found to be increased from ∼80 to ∼100 ml/kg (Hurtado, 1964; Sanchez et al., 1970). Results on sojourners to high altitude indicate that, similar to training, the increase in tHb and blood volume is also slow requiring weeks to months of high altitude exposure. At high altitude, the increase may be masked by a decrease in PV (Reynafarje et al., 1959). Therefore, a short-term stay at moderate and high altitude will not increase tHb and tEV (Myhre et al., 1970). A summary of 14 different studies Sawka et al. (2000) shows that several studies found no change in tEV upon ascent whereas some did, and explained discrepancies with the difference in the duration of exposure to high altitude. A gain in tEV between 62 and 250 ml/week was found when the sojourn lasted about 3 weeks.

Based on the raise in tEV upon ascent to high altitude and by training in normoxia it was concluded that effects of training and high altitude exposure on tHb might be additive, and that training at simulated altitude or by ascent to moderate or high altitude should cause an even further increase than training in normoxia. However, results are inconsistent ranging from no effect (Svedenhag et al., 1997; Friedmann et al., 1999) to a pronounced increase after 3–4 weeks of training at altitudes between 2100 and 2400 m (Levine and Stray-Gundersen, 1997; Friedmann et al., 2005; Heinicke et al., 2005). Lack of effects has in part been explained with lower training intensities at high than at low altitude, which is due to the decrease in performance with

increasing altitude (Cerretelli and DiPrampero, 1985). Several strategies have been developed aimed at improving the training efficiency while still "consuming" adjustments to hypoxia, one being the "sleep-high-train-low" protocol. Current concepts and concerns are reviewed in (Richalet and Gore, 2008; Stray-Gundersen and Levine, 2008; Robach and Lundby, 2012). Results are unclear, and often show no effect on tHb [e.g., in a well-designed, Placebo-controlled study by Siebenmann et al. (2012)]. A thorough analysis reveals that more than 14h per day of exposure to hypoxia seem to be required to attain a detectible increase in tHb and tEV (analysis in Schmidt and Prommer, 2008).

Control of erythropoiesis

It has been recognized by Bert (1882) that live at high altitude corresponds with increased hemoglobin, and later that Hct, Hb, and tHb are increased (Reynafarje et al., 1959; Hurtado, 1964; Sanchez et al., 1970), which was later recognized to be associated with elevated levels of erythropoietin (Mirand and Prentice, 1957; Scaro, 1960; Siri et al., 1966). The elevated tEV is thought to compensate for the decreased arterial O_2-content when the inspired PO_2 is low. Stimulation of vascularization by the vascular endothelial growth factor, VEGF, is another means warranting tissue O_2 supply in chronic hypoxia (for review see e.g., Marti, 2005). Both processes depend on sensing hypoxia within typical target cells and specific signaling pathways that adjust the expression of specific genes.

One such oxygen dependent mechanism is the control of expression by hypoxia inducible factors, HIF (Semenza, 2009). Active HIF consists of alpha and beta subunits. The beta subunit (HIF-β, also called ARNT) is expressed constitutively and is not directly affected by oxygen levels (Semenza, 1999). There are several isoforms of the alpha subunit, where HIF-1α seems to mainly control metabolic adjustments such as glycolysis (Hu et al., 2003), and HIF-2α has been identified as the major regulator of erythropoiesis (Scortegagna et al., 2005; Gruber et al., 2007). In hypoxia, the hydroxylation of HIF-alpha subunits by prolyl-hydroxylases (PDH) is prevented due to the lack of O_2 required as a direct substrate, which then prevents the hydroxylation-dependent poly-ubiquitinylation by the Van Hippel-Lindau tumor suppressor pVHL-E3 ligase and subsequent proteasomal degradation (Schofield and Ratcliffe, 2004) resulting in increased protein levels of HIF alpha subunits. Upon stabilization, alpha subunits enter the nucleus, where they dimerize with HIF-β. The dimer binds to a specific base sequence in the promoter region of genes called hypoxia response element, HRE, to induce the expression of genes (for recent reviews see (Semenza, 2009; Haase, 2010)). Besides stabilization, HIF-alpha subunits are also controlled at the transcriptional level (Görlach, 2009; Semenza, 2009).

In his review Haase (2010) nicely summarizes the experiments that led to the conclusion that HIF-2α is the major regulator of EPO production in liver (fetal) and kidney (adults), but that there are also a variety of different direct and indirect mechanisms. As shown in the scheme provided by Semenza (2009), although at that time related to actions of HIF-1α rather than HIF-2α, it can be seen that hypoxia controlled gene expression regulates not only the expression of EPO but also the expression of proteins whose action is a prerequisite for erythropoiesis such as EPO-receptors, iron transporters mediating intestinal iron reabsorption, and transferrin and transferrin receptors required for iron delivery to peripheral cells.

In the adult, the oxygen sensor controlling EPO production is in the kidney, where the cells producing EPO have been shown to be peritubular fibroblasts in the renal cortex (Maxwell et al., 1993; Eckardt and Kurtz, 2005). EPO production can be induced by two kinds of hypoxia: one is a decreased PO_2 in the kidney and in other tissues while the hemoglobin concentration is normal such as in hypoxic hypoxia. The other is called anemic hypoxia, where the hemoglobin concentration is decreased and but arterial PO_2 is normal resulting in a decreased venous PO_2 (Eckardt and Kurtz, 2005). There appears no difference in the effectiveness to produce EPO between these two situations. A mixture of these conditions might be a situation causing a decreased blood flow to the kidney at normal PO_2 and hemoglobin concentration, which should also result in decreased capillary and venous PO_2. The exact mechanisms controlling EPO production by the fibroblasts is not fully understood but appears to involve hypoxia-dependent recruitment of fibroblasts located in juxta-medullary and cortical regions (Eckardt et al., 1993).

EPO released into blood has many functions other than stimulating erythropoiesis (for review see Sasaki, 2003). In the bone marrow EPO binds to EPO receptors on progenitor cells in erythroblastic islands (Chasis and Mohandas, 2008), where it stimulates proliferation and prevents apoptotic destruction of newly formed cells (Lee and Percy, 2010). This increases the amount of red blood cells released from the bone marrow per time resulting in increased tEV when the rate of release exceeds red blood cell destruction (see above, sports anemia).

Effects of exercise and training on erythropoiesis

The increased tHb and tEV in trained athletes indicates that exercise stimulates erythropoiesis. An additional marker is the elevation of reticulocytes counts which can be observed within 1–2 days (Schmidt et al., 1988) after endurance (Convertino, 1991) and strength training units (Schobersberger et al., 1990). Despite apparent effects of single training units on red blood cell production several studies show that reticulocyte counts in athletes are not much different from sedentary controls (Lombardi et al., 2013) and values appear quite stable over years (Banfi et al., 2011; Diaz et al., 2011). There is, however, significant variation of reticulocyte counts in athletes during the year showing in general higher reticulocyte counts at the beginning of a season but lower values after intensive training sessions, competitions, and at the end of a season (Banfi et al., 2011). Nevertheless, markers of premature forms of reticulocytes are increased in athletes, which is indicative of stimulated bone marrow (Diaz et al., 2011; Jelkmann and Lundby, 2011).

Whereas the control of erythropoiesis in hypoxic and anemic hypoxia is well-understood, the signals stimulating erythropoiesis upon training in normoxia are unclear. Exposure to hypoxia causes a fast increase in EPO (Eckardt et al., 1989), but no or only minor changes in EPO have been observed after exercise of different modalities in untrained and trained individuals (Schmidt et al., 1991; Bodary et al., 1999), whereas the time course of

change in reticulocyte count is similar to effects of high altitude (Schmidt et al., 1988; Mairbäurl et al., 1990). The higher reticulocyte counts, a decreased mean red blood cell buoyant density and mean corpuscular hemoglobin concentration, and increased levels of other markers of a decreased mean red blood cell age (higher 2,3-DPG and P_{50}, higher red blood cell enzyme activities and creatine) have been found in peripheral blood from trained individuals (Mairbäurl et al., 1983; Schmidt et al., 1988), which are all indicators of an increased red blood cell turnover (Schmidt et al., 1988; Smith, 1995) and thus stimulated erythropoiesis. These newly formed red blood cells ease the passage of blood through capillaries because of a higher membrane fluidity and deformability of (Kamada et al., 1993).

Arguments for hypoxia as the relevant trigger for exercise induced erythropoiesis are sparse, and are at best indirect. Even during heavy exercise there is only a small decrease in arterial PO_2 (see chapter 2, arterial O_2 loading) that by itself will barely be sufficient to cause relevant renal EPO production. There is, however, a considerable decrease in renal blood flow with increasing exercise intensity that decreases renal O_2 supply (for an excellent review on splanchnic blood flow regulation in exercise see Laughlin et al., 2012). The O_2 supply to renal tubules might be further decreased, because renal cortical arteries and veins run parallel allowing exchange diffusion of O_2 that may cause arterial deoxygenation. PO_2 in cortical veins is low because of the high oxygen consumption required for Na^+ and water reabsorption of renal cortical epithelial cells (Eckardt and Kurtz, 2005). It can therefore be speculated that the decreased flow during exercise further decreases renal cortical PO_2 to a level causing significant hypoxia of the peritubular, EPO producing fibroblasts during exercise, and that this effect is aggravated as exercise intensity increases. Interestingly, training attenuates the decrease in renal blood flow, which seems more pronounced following endurance than high-intensity interval sprint training in rats (Musch et al., 1991; Padilla et al., 2011), which might explain the weak erythropoietic response in highly trained athletes.

A variety of humoral factors known to affect erythropoiesis also change during exercise. Androgens are long known for their stimulatory effect on erythropoiesis by stimulation of EPO release, increasing bone marrow activity, and iron incorporation into the red cells, which is best indicated by polycythemia as a consequence of androgen therapy (Shahidi, 1973; Shahani et al., 2009). Endurance exercise and resistance training cause a transient increase in testosterone levels in men and women (Hackney, 2001; Enea et al., 2009). Post-exercise values vary with exercise intensity in both genders. Interestingly, post-exercise testosterone levels also directly change with mood (win vs. loss), which seems more pronounced in men than women (for review see Shahani et al., 2009).

Stress hormones such as catecholamines and cortisol stimulate the release of reticulocytes from the bone marrow and possibly also enhance erythropoiesis (Dar et al., 2011; Hu and Lin, 2012). Erythropoiesis is also stimulated by growth hormone and insulin-like growth factors (Kurtz et al., 1988; Christ et al., 1997) which also increase during exercise (Hakkinen and Pakarinen, 1995; Schwarz et al., 1996).

HEMORHEOLOGY

Hematocrit not only affects the amount of O_2 that can be carried per volume of blood but also affects the rheological properties of blood. Due to its composition of plasma and blood cells it behaves as a non-Newtonian fluid, whose inner viscosity is affected by the shear forces and is determined by the concentration of plasma proteins (plasma viscosity), the physico-chemical properties of the red blood cell plasma membrane (deformability) and cellular hemoglobin concentration (cytosolic viscosity), the flow velocity (aggregation), and temperature (for review see El-Sayed et al., 2005). A high blood viscosity causes a high resistance to flow, increases the power output of the heart at a given cardiac output, and might impair local blood flow.

Because of the axial migration of blood cells when blood is moved with a high velocity it has been argued that plasma viscosity is the major determinant of whole blood viscosity (Rand et al., 1970). It is determined by the concentration of plasma proteins. The effect of altered hematocrit on blood viscosity is less clear. *In vitro*, a linear relation between blood viscosity and hematocrit values between 20% and 60% has been reported, when the shear stress is low (Chien et al., 1966), which is likely due to aggregation of red blood cells (Chien et al., 1967). Aggregation varies inversely with the flow velocity (Loewe and Barbenel, 1988) and is favored by fibrogen and immune-globulins binding to the red blood cells, whereas a role for albumin is less clear (Reinhart and Nagy, 1995). The high deformability of the red blood cells facilitates blood flow even at high hematocrit, particularly in the microcirculation. In fact, improved deformability contributes to the decrease in viscosity at high shear rates (El-Sayed et al., 2005). In contrast, increased osmolality decreases deformability by an increase of internal viscosity and altered surface-to-volume ratio although effects are small in the physiological range of changes in red blood cell hemoglobin concentration (Mohandas et al., 1980). Deformability of red blood cells has a temperature optimum in the physiological range and decreases significantly at temperatures below 35°C and above 45°C (Hanss and Koutsouris, 1984), which seems to be mainly determined by the lipid composition of the plasma membrane (Heath et al., 1982), whereas variations of the intracellular hemoglobin concentration within the physiological range do not affect deformability (Mohandas and Chasis, 1993).

Exercise and training affect all of the above mentioned determinants of whole blood viscosity. There is a well-documented increase in whole blood viscosity during exercise which reverses rapidly (for review see El-Sayed et al., 2005). It is mostly due to hemo-concentration and dehydration (Platt et al., 1981; Galea and Davidson, 1985; Vandewalle et al., 1988; Geor et al., 1994; Yalcin et al., 2000). Results on exercise induced changes in the deformability of red blood cells are divergent and indicate decreased (most reports; e.g., Platt et al., 1981; Geor et al., 1994; van der Brug et al., 1995; Bouix et al., 1998; Smith et al., 2013), unchanged (Neuhaus et al., 1992), and increased deformability (Gurcan et al., 1998) (for review see El-Sayed et al., 2005). Exercise in hypoxia aggravated the adverse effect on deformability, which was associated with decreased actin and spectrin content and down-regulation of other proteins, and enhanced the response of red blood cells to oxidative stress (Mao et al.,

2011). However, the exercise-induced decrease in deformability appears to be independent of oxidants produced by shear stress, because it was not prevented by strong antioxidant prophylaxis (Kayatekin et al., 2005). Studies may be hampered by the fact that PV and osmolality changes may revert rapidly depending on exercise duration and intensity indicating the proper choice of time-points for sampling when blood is collected after rather than during the exercise. Ernst and colleagues nicely report during and after exercise kinetics of blood viscosity and show that deformability of erythrocytes is increased during and normalized within a few hours after exercise (Ernst et al., 1991). The increase in blood lactate during exercise seems not to affect deformability (Simmonds et al., 2013), neither does lactate affect aggregation (Connes et al., 2007). However, there are indications that high lactate impairs deformability in untrained but improves in trained individuals (Connes et al., 2004). Training might decrease blood viscosity by improving the deformability of red blood cells. The membrane fluidity of red blood cells was increased in sprinters and long distance runners (Kamada et al., 1993), which is consistent with the finding that the exercise-induced decreased in deformability was found to be attenuated by training (Ernst, 1987; Yalcin et al., 2000). This might be explained by the higher deformability of newly formed red blood cells (Mairbäurl et al., 1983; Linderkamp et al., 1993). Erythropoietin, which was found slightly elevated (see above) seems to be favorable (Pichon et al., 2013; Zhao et al., 2013), probably by decreasing the mean red blood cell age and young red blood cells having an improved membrane flexibility (Mohandas and Chasis, 1993). In contrast, insulin-like growth factors and growth hormone seem to increase viscosity (Monnier et al., 2000; Connes et al., 2007). In summary, most studies show improved rheological properties of blood in trained individuals (see meta-analysis by Romain et al., 2011).

Together these results indicate that the increase in whole blood viscosity during exercise is caused by the combined effects of increased plasma viscosity and decreased deformability of the red blood cells, and potentially impairs microcirculation and thus O_2 delivery to working muscle. Moderation of this effect might be brought about by NO released from endothelium and red blood cells with increased shear stress, because nitrosylation of cytoskeletal proteins in the red blood cell membrane seems to improve deformability (Grau et al., 2013). In contrast, training seems to increase deformability and to decrease whole blood viscosity in support of tissue oxygenation.

RED BLOOD CELL MEDIATED VASODILATION

Precise control of regional blood flow is required to match substrate demand and removal of metabolites, which is of particular importance when the metabolic activity is high such as in exercising skeletal muscle. Nitric oxide (NO) is an important signaling molecule that causes local vasodilation. It is typically formed in vascular endothelial cells upon a variety of stimuli, the most important during exercise likely being shear stress (Pohl et al., 1986; Shen et al., 1995). Hemoglobin has been shown to tightly bind NO to form nitrosylhemoglobin (Hb-cys-NO; SNO-Hb) in an O_2 saturation dependent manner with higher affinity for deoxyhemoglobin, a reaction that also causes formation of Met-Hb (Gow and Stamler, 1998; Grubina et al., 2007). Binding has been interpreted as a sink for NO produced by endothelium to prevent exaggerated and wide-spread vasodilation. However, it has also been hypothesized that Hb not only binds but also releases and/or produces NO from SNO-Hb to cause local vasodilation (Robinson and Lancaster, 2005).

It has been shown experimentally that NO released from red blood cells causes vasodilation when the shear stress is increased and when the tissue is made hypoxic (Ulker et al., 2013). Red blood cells produce bioactive NO equivalents in an O_2 saturation, pH, and redox-state dependent manner, which appears to be an allosteric, autocatalytic reaction with characteristics of a nitrite reductase reaction (for review see Gladwin and Kim-Shapiro, 2008). When nitrite is added to fully deoxygenated Hb, NO is released and Met-Hb is formed (Gladwin and Kim-Shapiro, 2008). Bioactivity is indicated by the notion that upon nitrate infusion, NO binding to hemoglobin and vasodilation are tightly coupled and are favored by hypoxia (Crawford et al., 2006). Kleinbongard et al. (2006) presented immunehistochemical and functional evidence of the presence of an endothelial NO-synthase type of enzyme in human and mouse red blood cells indicating the potential to produce NO from L-arginine. It is unclear, however, whether this reaction is active in controlling microcirculation in working skeletal muscle (which generates a low oxygen environment because of its requirement for oxygen).

ATP in plasma is another stimulus for endothelial NO production (Sprague et al., 1996). ATP is released from many cells where it modifies a variety of functions (Praetorius and Leipziger, 2009). Local vasodilation has been shown to depended on the presence of red blood cells (Dietrich et al., 2000). Thus, it has been hypothesized that red blood cells release ATP and cause an NO-dependent increase in blood flow (Gonzalez-Alonso et al., 2002). ATP release is not only an *in vitro* phenomenon but has also been demonstrated vivo, where elevated ATP has been found in the venous effluent from exercising forearm muscle (Forrester, 1972; Ellsworth et al., 1995). This effect was even enhanced when exercise was performed in hypoxia (Gonzalez-Alonso et al., 2002).

The major stimulus for ATP release from red blood cells seems to be mechanical deformation (Sprague et al., 1996; Ellsworth et al., 2009), where ATP release seems to depend on the shear rate (Mairbäurl et al., 2013). Also *in vitro* hypoxia stimulates the release of ATP from red blood cells (Bergfeld and Forrester, 1992). Futhermore, hypoxia greatly enhances ATP release induced by shear stress indicating that effects are additive (Mairbäurl et al., 2013). Other stimulators of ATP release from red blood cells are beta adrenergic stimulators and prostacyclin (Olearczyk et al., 2001), and an increase in temperature (Kalsi and Gonzalez-Alonso, 2012). The exact release mechanism is unclear. An involvement of CFTR has been discussed (Sprague et al., 1998) but it is unclear whether CFTR is actually present in human red blood cells. A variety of other mechanisms for ATP release have been described (for review see Praetorius and Leipziger, 2009), some of which seem to involve pannexin1- (Qiu and Dahl, 2009; Qiu et al., 2011). Intravascular hemolysis seems not to contribute significantly to ATP release from erythrocytes exposed to shear-stress (Mairbäurl et al., 2013).

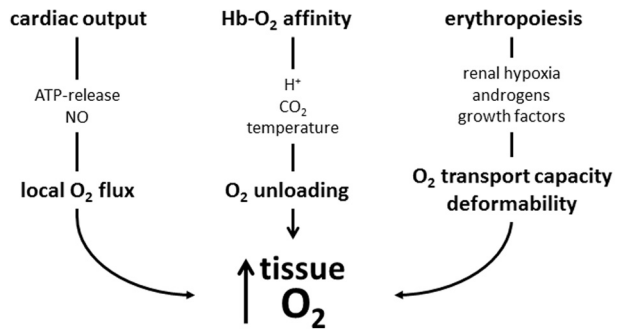

FIGURE 3 | Schematic presentation of mechanisms increasing muscle oxygen supply acutely during exercise and by training discussed in this review. During exercise local blood flow is increased by mediators causing local vasodilation, which is supported by red blood cell-mediated NO production. Acidosis, CO_2 and hyperthermia decrease Hb-O_2-affinity and enhance O_2 release from its bond to hemoglobin. These improvements may in part be blunted by increased blood viscosity (not shown in scheme). Training stimulates erythropoiesis to increase the O_2-transport capacity. The newly formed cells also have an improved deformability which facilitates muscle blood flow. Training also increases red blood cell 2,3-DPG (not shown), which further enhances O_2 release from Hb.

Taken together these results indicate that red blood cells support local vasodilation in tissues with a high O_2 demand by directly mediating NO release and enzymatic production and by release of ATP, which causes NO release from endothelial cells by mechanisms, which are greatly enhanced in exercise when shear stress is increased by increased blood flow, O_2 is low due to increased consumption, and the increase in temperature.

CONCLUSION

There are many mechanisms that contribute to an increased tissue oxygen supply during exercise. **Figure 3** summarizes those, where red blood cell are involved. They involve adjustments during exercise and to training. During exercise the increased O_2 demand of skeletal muscle is mainly matched by increasing muscle blood flow by increasing cardiac output, by modulating blood flow distribution among active and inactive organs, and by optimizing microcirculation (Laughlin et al., 2012). Red blood cells support local blood flow by providing the vasodilator NO by direct conversion from nitrate and by release of ATP causing endothelial NO release. At any given capillary blood flow the amount of O_2 unloaded from Hb to the cells of working muscle can be increased greatly by decreasing Hb-O_2 affinity. This happens as the cells enter the capillaries supplying the muscle cells, where they are exposed to increased temperature, H^+ and CO_2. Training further enhances O_2 flux to the working muscle at all levels of regulation: It increases maximal cardiac output, improves blood flow to the muscles by stimulating vascularization, and improves the rheological properties of red blood cells. Training increases total hemoglobin mass by stimulating erythropoiesis, which increases the amount of O_2 that can be carried by blood. It also increases red blood cell 2,3-DPG, which increases the sensitivity of Hb-O_2 affinity to acidification dependent O_2-release. The system appears to be optimized for exercise at low altitude, because in an hypoxic environment the decreased arterial PO_2, which is the major determinant for O_2 diffusion, cannot be compensated adequately by the above mentioned O_2 transport mechanisms resulting in a decrease in performance with increasing degree of hypoxia (Cerretelli and DiPrampero, 1985).

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Red blood cell vesiculation in hereditary hemolytic anemia

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Hereditary hemolytic anemia encompasses a heterogeneous group of anemias characterized by decreased red blood cell survival because of inherited membrane, enzyme, or hemoglobin disorders. Affected red blood cells are more fragile, less deformable, and more susceptible to shear stress and oxidative damage, and show increased vesiculation. Red blood cells, as essentially all cells, constitutively release phospholipid extracellular vesicles *in vivo* and *in vitro* in a process known as vesiculation. These extracellular vesicles comprise a heterogeneous group of vesicles of different sizes and intracellular origins. They are described in literature as exosomes if they originate from multi-vesicular bodies, or as microvesicles when formed by a one-step budding process directly from the plasma membrane. Extracellular vesicles contain a multitude of bioactive molecules that are implicated in intercellular communication and in different biological and pathophysiological processes. Mature red blood cells release in principle only microvesicles. In hereditary hemolytic anemias, the underlying molecular defect affects and determines red blood cell vesiculation, resulting in shedding microvesicles of different compositions and concentrations. Despite extensive research into red blood cell biochemistry and physiology, little is known about red cell deformability and vesiculation in hereditary hemolytic anemias, and the associated pathophysiological role is incompletely assessed. In this review, we discuss recent progress in understanding extracellular vesicles biology, with focus on red blood cell vesiculation. Also, we review recent scientific findings on the molecular defects of hereditary hemolytic anemias, and their correlation with red blood cell deformability and vesiculation. Integrating bio-analytical findings on abnormalities of red blood cells and their microvesicles will be critical for a better understanding of the pathophysiology of hereditary hemolytic anemias.

Keywords: microvesicle, red blood cell, hemolytic anemia, membrane disorder, enzyme disorder, hemoglobinopathy, erythrocyte

INTRODUCTION

Red blood cells (RBCs) are the most abundant cell type in human blood and they function to transport oxygen (O_2) from the lung to all tissues and cells, and to transport carbon dioxide (CO_2) from tissues back to the lung. These functions dictate the unusual capacity of RBCs to pass through all types of vessels, and even to squeeze in capillaries of smaller diameters than RBCs themselves (Mokken et al., 1992). Indeed, the molecular structures of normal RBC membrane, cellular content, and energy machinery enable such extraordinary deformability under the high shear forces of blood flow (Svetina, 2012). Many patients with hereditary (inherited) hemolytic anemias show aberrant RBC deformability due to defects in one or more of RBC molecular components which are crucial for the mechanical strength of RBCs as well as their protection from oxidative stress (Hebbel, 1991; Gurbuz et al., 2004; Da Costa et al., 2013). Non-immune hereditary hemolytic anemias may be classified according to the underlying defects into three major groups: membranopathies, enzymopathies, and hemoglobinopathies (Dhaliwal et al., 2004).

Hereditary spherocytosis (HS) is the most common type of hereditary hemolytic anemia. The estimated prevalence of this membrane disorder is 1 in 5000 in the white population of Northern Europe. Red blood cell glucose-6-phosphate-dehydrogenase (G6PD) deficiency is the most common enzyme disorder worldwide, affecting 420 million of the world population. Less common is hemolytic anemia due to pyruvate kinase (PK) deficiency with an estimated prevalence of 1 in 20,000 in the white population. Sickle cell anemia is the conspicuous example of anemia due to a hemoglobinopathy. Its prevalence is 1–5 in 10,000. For more prevalence data and other hereditary hemolytic anemias, the ENERCA (European NEtwork for Rare and Congenital Anaemias) website (www.enerca.org) and the portal for rare diseases and orphan drugs (www.orpha.net) are recommended.

Both in physiological and pathophysiological processes the role of extracellular vesicles is increasingly appreciated. Most cells, if not all, secrete extracellular vesicles, which comprise heterogeneous populations of vesicles of different compositions and physicochemical properties. Considerable evidence is accumulating showing the significance of extracellular vesicles as key players for intercellular communication, in health and disease (EL Andaloussi et al., 2013). Focusing on RBC vesiculation, both *in vivo* or under blood storage conditions *ex vivo* mature RBCs lose their membranes through shedding of microvesicles, a class

of extracellular vesicles defined by the fact that they originate from the plasma membrane (Greenwalt, 2006). In hereditary hemolytic anemias, the molecular defects affect not only the RBC but also their normal vesiculation pattern, resulting in the release of circulating microvesicles which probably have a different composition compared to those derived from normal RBCs. Loss of RBC membrane as microvesicles likely alters the cell's surface area-to-volume (S/V) ratio and RBC internal viscosity, and hence, perturbs RBC deformability (Mohandas et al., 1980).

Alterations in RBC deformability can be measured using a laser diffraction technique known as ektacytometry. Using this technique, a thin layer of RBCs is sheared between two rotating surfaces, transforming RBCs from the discoid morphology into the elliptical one. The laser beam is deflected by RBCs to produce patterns from which RBC deformability is assessed (Mohandas et al., 1980). Ektacytometry is a robust and easy-to-perform technique, which can be routinely used to scan blood samples to provide valuable information about abnormalities of RBC deformability (Vent-Schmidt et al., 2013). Harnessing RBC deformability and the emerging findings in extracellular vesicle field may open up new avenues for understanding and diagnosing rare, possibly neglected, diseases like hereditary hemolytic anemias. This review provides brief insights into vesiculation, RBC-derived vesicles and RBC deformability while emphasizing their translational value for patients with hereditary hemolytic anemias.

EXTRACELLULAR VESICLES AND THEIR PATHOPHYSIOLOGICAL SIGNIFICANCE

Intercellular communication was believed to occur only via cell-to-cell contact and/or secreted soluble factors. Within the last three decades, there has been a paradigm shift in studying extracellular vesicles as key mediators of intercellular communication. Extracellular vesicles are membranous lipid bilayer-vesicles ubiquitously secreted by different cell types. Although there are conserved vesicular components, the composition of extracellular vesicles considerably varies according to the secreting cells, the stimulus for their formation, in addition to the inter-individual variability (Thery et al., 2009; Bastos-Amador et al., 2012). Extracellular vesicles may be classified by their intracellular origins. For instance, a subtype of extracellular vesicles known as exosomes originate from multi-vesicular bodies, and they are secreted by a two-step process: inward budding of the plasma membrane to form multivesicular bodies (MVBs) followed by fusion of the MVBs with the plasma membrane. The second subtype of extracellular vesicles is known as microvesicles or ectosomes, which are released by outward budding from the plasma membrane (Thery et al., 2009).

Over the last two decades, extracellular vesicles have been intensively studied after finding that they are more than cellular artifacts or clearance machineries of cellular junk. Stegrnayr and Ronquist have published the first report on the functionality of extracellular vesicles, showing that prostasomes could promote human sperm motility (Stegmayr and Ronquist, 1982). However, the biological functions of extracellular vesicles remained underestimated and unanalyzed until 1996 when Raposo et al. reported that B lymphocytes-derived exosomes could stimulate adaptive immune responses (Raposo et al., 1996). This work stimulated the scientific community to investigate the biological functions of extracellular vesicles, especially that these vesicles are secreted by nearly all cell types, including stem cells, cancer cells, and cellular components of blood. Beside their ubiquitous secretion, extracellular vesicle formation and release probably occurs in a tightly controlled manner and their bioactive cargo is selectively sorted (Kriebardis et al., 2012).

Recently, advances in analytical techniques for nucleic acids and proteomics enabled the identification of mRNAs, regulatory miRNAs, and functional proteins loaded into/onto extracellular vesicles. Such findings emphasize the pathophysiological significance of extracellular vesicles, which may transfer these bioactive payloads from one cell to another cell in many diseases (Raposo and Stoorvogel, 2013). Pioneering work was performed by Valadi et al. who demonstrated that exosomes could transfer functional mRNA and miRNAs between cells (Valadi et al., 2007). Later, Skog et al. found that glioblastoma-derived microvesicles could promote tumor growth and angiogenesis via delivering angiogenic and invasiveness factors to recipient cells in the tumor microenvironment (Skog et al., 2008). Moreover, a growing body of findings shows the implications of extracellular vesicles that may fuel bacterial or viral virulence through transferring pathogenic factors, either in pathogen-derived vesicles or in vesicles released from pathogen hijacked-host cell vesiculation machineries (Silverman and Reiner, 2011). Very recent work shows the importance of circulating exosome-like vesicles released by *Plasmodium falciparum*-infected RBCs to the sexual stage of malarial infection (Regev-Rudzki et al., 2013).

RED BLOOD CELL-DERIVED VESICLES

Red blood cells constitute 40% of the total blood volume and they are one of the major vesicle-secreting cells in the circulating blood (Xiong et al., 2012). Although the majority of circulating microvesicles in healthy individuals are derived from platelets, RBCs-derived microvesicles levels are elevated during the course of some pathological conditions such as malaria and sickle cell disease (Barteneva et al., 2013). Interestingly, extracellular vesicles were first discovered as released by blood cellular components. In 1967, extracellular vesicles were described by Wolf as products or dust that resulted from platelet membrane fragmentation in human plasma (Wolf, 1967). Later, in 1983, two independent groups reported the release of extracellular vesicles by exocytosis of multivesicular endosomes during the maturation of reticulocytes (Harding et al., 1983; Pan and Johnstone, 1983). Although reticulocytes, or immature RBCs, secrete exosomes during the remodeling process that accompanies their maturation, mature RBCs are probably the only cells that do not secrete exosomes and shed only microvesicles (de Vooght et al., 2013).

Red blood cell-derived microvesicles are submicron membranous structures with a lipid bilayer rich in different phospholipids and proteins, which are derived from parental RBCs. Their average size is approximately 100–200 nm.

During their 120-days life span, normal RBCs lose approximately 20% of their hemoglobin and membrane through vesiculation (Werre et al., 2004). Red blood cell vesiculation can be increased *in vitro* by increasing levels of intracellular calcium and

by depleting endogenous ATP (Lutz et al., 1977; Bevers et al., 1992). Under blood storage conditions, RBCs undergo structural and morphological changes associated with ATP depletion and oxidative damage of membrane lipids and proteins. Such changes, known as storage lesions, eventually result in shedding microvesicles and affect RBC survival after blood transfusion (Bosman et al., 2008). These lesions are dependent on storage conditions, storage length, and additives used in blood products (Veale et al., 2011).

Red blood cell membrane components are key players in microvesicle formation and release, and hence acknowledging their roles may improve our understanding for RBC vesiculation. The RBC membrane is composed of a phospholipid bilayer, which is penetrated by integral proteins (e.g., band 3, glycophorins A and C, Rhesus-associated antigen), and tethered to the cytoskeleton network composed of α- and β-Spectrin and actin via protein 4.1R, protein 4.2, and ankyrin (**Figure 1**). The RBC membrane and cytoskeleton proteins function to maintain the integrity of plasma membrane, and also to confer deformability and mechanical strength to RBCs (Mohandas and Gallagher, 2008). Besides, several RBC membrane proteins have transport functions. For instance, glucose transporter 1 (GLUT1) and aquaporin-1 controls transport of glucose and water, respectively. Also, multiple ion channels span the RBC membrane to regulate ion gradients and the hydration state of RBCs (Thomas et al., 2011).

In the resting state, RBC membrane phospholipids are asymmetrically distributed across the bilayer, meaning that the neutral choline-containing phospholipids, like phosphatidylcholine and sphingomyelin, reside in the outer leaflet of the RBC while the charged amino-containing phospholipids, like phosphatidylserine (PS), phosphatidylinosirol, and phosphatidyl-ethanolamine, reside in the inner leaflet of the membrane (Zwaal and Schroit, 1997). Beside the RBC cytoskeleton, the asymmetry of phospholipids across the RBC membrane is crucial for maintaining RBC stability, deformability and mechanical strength (Manno et al., 2002). The asymmetric distribution of phospholipids is tightly controlled by three key enzymes. The first is an ATP-dependent flippase which pumps the amino-containing phospholipids from the outer leaflet to the inner leaflet. The second is an ATP-dependent floppase which controls the reverse transfer of the choline-containing phospholipids from the inner leaflet to the outer leaflet, at slower rate compared to flippases. The third enzyme is a divalent-cation activated scramblase which controls the bi-directional transfer of phospholipids down their concentration gradients to achieve a more symmetrical distribution of phospholipids (Graham, 2004). Disruption of the plasma membrane phospholipid asymmetry and externalization of PS are major steps in RBC vesicle formation and release (Graham, 2004) (**Figure 2**).

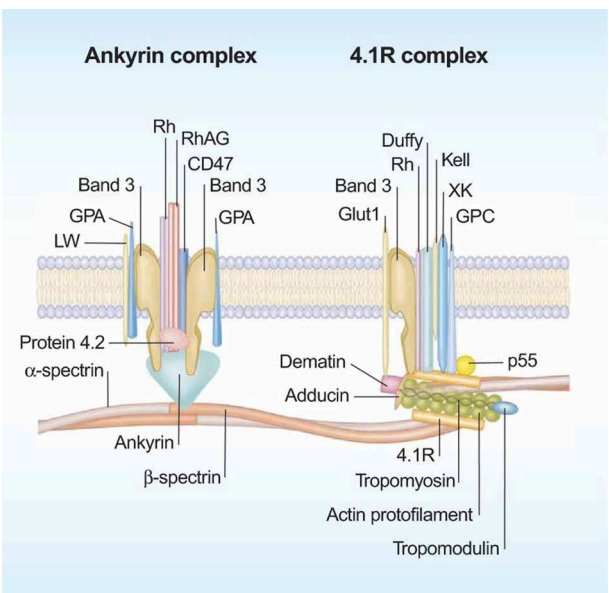

FIGURE 1 | Schematic representation of the red blood cell membrane. The red blood cell membrane consists of a phospholipd bilayer that is anchored to the 2-dimensional elastic network of skeletal proteins (mainly spectrin) through transmembrane proteins (reproduced with permission from Mohandas and Gallagher, 2008).

High levels of intracellular Calcium activate scramblase and inhibit flippase, and that results in a collapse of lipid asymmetry and externalization of PS. The role of the floppase enzyme on disturbing the membrane lipid asymmetry is not clear. However, it may be involved in the rapid translocation of PS to the external leaflet (Bevers et al., 1999; Graham, 2004). Meanwhile, the increase of intracellular calcium also activates proteolytic enzymes like calpain which breaks down the tethering points between the membrane cytoskeleton and the plasma membrane, facilitating the release of microvesicles (Morel et al., 2011). However, Knowles et al. reported that RBC cytoskeleton did not break down but retracted to the cell body during membrane deformation under shear stress (Knowles et al., 1997). This finding may explain the release of microvesicles, which are free from cytoskeletal components. A simulation study for RBC membrane demonstrated that the cell cytoskeleton may break and reform in a dynamic manner and this may confer solid-to-fluid transition characteristics to the RBC membrane under different stimuli (Li et al., 2007). It is noteworthy that both the calcium-induced phospholipid scrambling and the microvesicle release may occur in two independent pathways. Bucki et al. found that stabilizing the phospholipid asymmetry by spermine did not inhibit calcium-induced RBC vesiculation (Bucki et al., 1998). Yet the exact underlying molecular mechanisms of RBC vesiculation are far from being completely deciphered. It is not well known how different stimuli, like calcium influx, amphiphiles, shear stress, or ATP depletion, may induce shedding of microvesicles that have different compositions. Furthermore, the selective sorting of certain membrane components into microvesicles remains to be understood (Knowles et al., 1997). Although the blood storage remodeling process is presumed to be similar to RBC aging *in vivo*, the role of cytoskeleton proteins in vesicle formation may be different *in vitro* compared to *in vivo* (Bosman et al., 2010; Canellini et al., 2012). Moreover, RBCs of different phenotypes may shed microvesicles differently. For instance, although RBCs in Scott syndrome, a rare hereditary bleeding disorder, have a normal morphology and show no abnormalities in cytoskeleton composition, they resist vesiculation upon stimulation with the

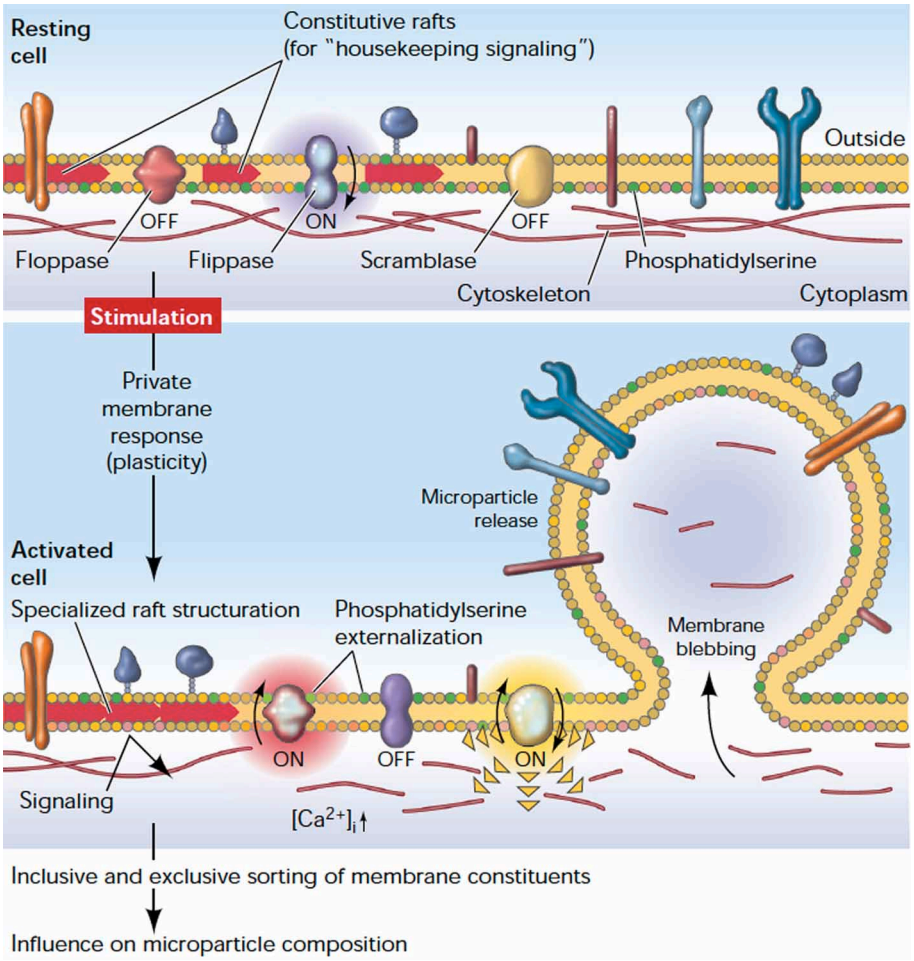

FIGURE 2 | The plasma membrane response to cell stimulation. Depicted is a general plasma membrane: a well-structured entity characterized by a controlled transverse distribution of lipids and different kinds of (transmembrane) proteins, and laterally organized domains (rafts). Stimulation causes a redistribution of proteins, structuration of rafts, externalization of PS, and release of microparticles (reproduced with permission from Hugel et al., 2005).

Calcium ionophore A23187 (Bevers et al., 1992). In a nutshell, RBCs derived microvesicles are not always equal, and both RBC phenotype and the triggering stimulus may determine the characteristics of the released microvesicles.

Several physiological and pathophysiological functions of RBC-derived microvesicles have been described. The main characteristic of RBCs-derived microvesicles is the externalization of the negatively charged PS, which has two main physiological implications: (1) Promoting coagulation; (2) Acting as an "eat me" signal for macrophages. The procoagulant activity of PS relies on the catalytic activity of PS. PS acts as a binding surface for prothrombinase and other coagulation factors, increasing the transformation rate of prothrombin into thrombin, a major coagulation molecule (Lentz, 2003). Although platelet-derived microvesicles play an essential role in haemostasis regulation, RBC-derived microvesicles could be equally important in certain pathological conditions like sickle cell disease (Tan and Lip, 2005; Weiss et al., 2011). Additionally, RBC-derived microvesicles may promote thrombus formation *in vivo* via exposing tissue factor, a key initiator of coagulation (Biro et al., 2003). Exposed PS is a proposed "eat-me" signal for phagocytes to engulf PS-exposing apoptotic cells. Such signaling provides a protection from the toxic effects of apoptotic cells (Chaurio et al., 2009). The same clearance mechanism may occur for PS-exposing microvesicles. It has been proposed that RBC vesiculation may serve as a self-protection mechanism to prevent the early removal of RBCs through removing the damaged components of the RBC membrane. Thus, RBC-derived microvesicles "sacrifice" themselves; the reticuloendothelial system removes the PS-exposing microvesicles instead of their parental RBCs, which probably have restored lipid asymmetry after vesiculation (Willekens et al., 2008).

Red blood cell-derived microvesicles may also be implicated in immunomodulation, nitric oxide (NO)–redox homeostasis, and in inflammatory reactions. These microvesicles may have immunosuppressive activities by down-regulating the macrophages that uptake them. Sadallah et al. showed that RBCs-derived microvesicles were taken up by macrophages and

this inhibited the release of tumor necrosis factor-α (TNF-α) and interleukin 8 when these macrophage were exposed to zymosan A and lipopolysaccharide (LPS), well-recognized as macrophage stimulators (Sadallah et al., 2008). Such immune-suppressive activities may have implications in the post-transfusion immune-suppression and infections. On the other hand, Mantel et al. found that RBCs infected with *P. falciparum* could release microvesicles rich in host and parasites proteins and these microvesicles possessed a potent stimulatory effect on cells of the innate immune system (Mantel et al., 2013).

During blood storage, RBCs are subjected to storage lesions which results in reduced integrity of the RBC membrane, associated with releasing the RBC oxy-hemoglobin content as cell-free oxy-hemoglobin and vesicular oxy-hemoglobin. Both forms may have enhanced scavenging activities for NO, a potent vasodilator. Infusion of stored blood was shown to have a potent vaso-constrictive effect associated with the degree of storage-related hemolysis, potentially due to NO scavenging (Donadee et al., 2011). Regarding the potential pro-inflammatory activities of RBC-derived microvesicles, the vesicle membrane is a source for amino-containing phospholipids, which are substrates of the secretory phospholipase A2 (sPLA2) enzyme. Consumption of vesicular phospholipids by sPLA2 may results in production of lysophosphatidic acid, which is implicated in cell proliferation, migration and inflammatory reactions (Fourcade et al., 1995).

HEREDITARY HEMOLYTIC ANEMIA: UNDERSTANDING THE MOLECULAR BIOLOGY

Hereditary hemolytic anemias basically stem from intrinsic RBC disorders. In this review we focus on this group of disorders which can be due to mutations in genes that encode for: (1) a transmembrane/cytoskeleton protein; (2) an enzyme needed for a metabolic reaction in RBCs; or (3) hemoglobin. The phenotypes of hereditary hemolytic anemias range from being clinically asymptomatic to severe life threatening conditions. In this section we discuss the molecular bases of these disorders, and highlight their potential relationships with RBC vesiculation.

RED BLOOD CELL MEMBRANE DISORDERS

Red blood cell membrane proteins control the mechanical strength, the elastic deformability and the hydration state of RBCs. Defect in one or more of these proteins may lead to RBC membrane instability, increased membrane rigidity, and/or aberrant hydration state of RBCs. These perturbations often are associated with morphological changes and decreased life span of the RBCs.

Hereditary spherocytosis

Hereditary spherocytosis (HS) is the most common inherited hemolytic anemia due to a membrane defect. Inheritance is mostly autosomal dominant and peripheral blood smears of HS patients show spherocytes (sphere-shaped RBCs). However, spherocytic cells may also be seen in other acquired disorders like autoimmune hemolytic anemias, thermal injuries, and venom poisoning (Perrotta et al., 2008). The spherocytic morphology of RBCs is due to loss of membrane surface area relative to the cell volume. Most cases of HS are caused by deficiencies in the membrane or cytoskeleton proteins ankyrin, band 3, protein 4.2, β-spectrin, or α-spectrin. The clinical severity of HS varies according to the degree of spectrin loss, even if the primary defect is not in the gene encoding spectrin (Chasis et al., 1988; Eber et al., 1990). The aforementioned proteins are vital for the vertical tethering of the RBC cytoskeleton network with the membrane lipid bilayer, and their loss results in reduced mechanical strength and loss of membrane surface area through vesiculation. Deficiency of one or more of the cytoskeleton components may create an area of weakness in the membrane, facilitating RBC vesiculation. Alternatively, loss of one of the integral proteins, for instance band 3, may affect the membrane integrity. Thus, the released microvesicles may differ from those released due to deficiencies in cytoskeleton proteins. Indeed, in HS, RBC-derived microvesicles were found to have different compositions based on the underlying molecular defect. Reliene et al. showed that microvesicles released from spectrin/ankyrin-deficient RBCs were enriched in band 3 proteins. However, band 3-deficient RBCs did not lose their few band 3 proteins. Loss of band 3 in microvesicles released from spectrin/ankyrin-deficient RBCs reduced the binding of membrane-bound immunoglobulin G (IgG) to band 3 clusters on RBCs, and hence reduced opsonization and immune-mediated clearance of RBCs. In contrast, band 3-deficient RBCs did not lose their band 3 molecules, which remained attached to the cytoskeleton and clustered on the RBC membrane, and hence were opsonized by IgG *in vivo* (Reliene et al., 2002).

Hereditary elliptocytosis

Hereditary elliptocytosis (HE) is another diverse group of inherited RBC membrane disorders that are transmitted in an autosomal dominant manner with exception of pyropoikilocytosis (HPP), a severe form of HE, which is generally transmitted in an autosomal recessive manner. HE is characterized by elliptical RBCs (elliptocytes) on peripheral blood smears. However, elliptocytes may also be seen in thalassemia and megaloblastic anemias. The clinical manifestations of HE and HPP vary from silent asymptotic conditions to a severe life threatening hemolytic anemia (Nagel, 2006).

The hallmark of HE is the loss of mechanical stability of the RBC membrane due to defects in membrane proteins maintaining the lateral linkages of the RBC cytoskeleton. These defects include quantitative and/or qualitative defects in proteins α-spectrin, β-spectrin, and protein 4.1. The quantitative defects result from reduced expression of genes encoding for normal proteins. On the other hand, qualitative defects result in an alteration in the protein structure that affects the protein interaction with the adjacent proteins. HE RBCs are more susceptible to shear-stress induced fragmentation which is associated with membrane loss and progressive transformations of RBCs from the biconcave morphology to the elliptical or oval one. Membrane loss is potentially through shear-induced vesiculation, and such loss may be involved in decreased deformability of HE RBCs. However, the exact relationship between HE and vesiculation remains to be studied (Wagner et al., 1986; Gallagher, 2004).

Hereditary stomatocytosis

Hereditary stomatocytosis (HSt) is a genetic disorder that comprises a heterogeneous group of syndromes, in which the RBC

membrane is leaky to monovalent cations, showing increased permeability to Na^+ and K^+. HSt is inherited in an autosomal dominant manner. HSt RBCs can be seen as mouth-shaped (stoma-) RBCs in peripheral blood smears. Like other RBC membrane disorders, the phenotype of HSt patients may range from being asymptomatic to suffering from a severe hemolytic anemia. Membrane leakiness of monovalent cations alters the osmotic pressure across the RBCs membrane, and hence affects RBC volume and cellular deformability. Such changes may decrease the life span of defective RBCs and increase their splenic sequestration (Bruce, 2009; Da Costa et al., 2013).

HSt can generally be classified into two main phenotypes: overhydrated HSt (OHSt) and dehydrated DHSt (also known as xerocytosis). In OHSt, RBCs have high intracellular sodium concentration, which results in cell overhydration, reduction in the mean cell hemoglobin concentration (MCHC), and transformation of RBCs into spherotic non-deformable cells. In xerocytosis, increased membrane leakiness, in particular K^+,results in a reduction of total intracellular cation content. This induces RBC dehydration associated with elevated MCHC, and thus perturbs RBC deformability (Da Costa et al., 2013).

In OHSt, reduced levels of stomatin, a RBC membrane protein implicated in regulating K^+/Na^+transport across the membrane, have previously been found. However, Stewart and Turner reported that stomatin knockout mice did not have hemolytic anemia, which may indicate that stomatin deficiency in OHSt is a secondary event and not the primary underlying molecular defect of DHSt (Stewart and Turner, 1999). In agreement with this, mutations in anion exchanger 1 (band 3) (Bruce et al., 2005), the putative ammonium transporter Rhesus-associated glycoprotein (RhAG) (Bruce, 2009), and GLUT1 (Flatt et al., 2011) have recently been implicated in OHSt. Some patients with HSt show normal levels of stomatin. It is worth mentioning that stomatin is a major lipid raft enriched in microvesicles derived from normal RBCs during blood storage. Actin-stomatin tethering may regulate the vertical association between the membrane and cytoskeleton, and hence regulate vesiculation. Indeed, Wilkinson et al. found that calcium-induced vesiculation is perturbed in stomatin-deficient RBCs of OHSt patients. By using calcium and the ionophore A23187, microvesicles-derived from stomatin-deficient RBCs were shown to be different in shape, size, number and composition when compared to microvesicles derived normal RBCs (Wilkinson et al., 2008). Yet, vesiculation of stomatin-deficient RBCs under physiological conditions remains to be studied. Some families affected by DHSt were recently shown to have mutations in PIEZO1. PIEZO proteins are pore forming subunits of mechanically activated cation channels (Andolfo et al., 2013). PIEZO1 mutations may be involved in leakiness of RBC cations through the membrane (Albuisson et al., 2013). It is not clear if RBC vesiculation is directly affected by mutations in PIEZO1 or if it occurs as a secondary event due to the perturbed deformability.

HEREDITARY HEMOLYTIC ANEMIA DUE TO RED BLOOD CELL ENZYME DISORDERS

Mature RBCs lack nuclei and mitochondria, and they have limited metabolic activities compared to other cells in the human body. Nevertheless, their limited metabolic pathways are capable of maintaining the RBC viability during its 120-days life span. These pathways are also crucial for O_2 binding and delivery, through preserving hemoglobin in its functional form (Prchal and Gregg, 2005). Three main metabolic pathways exist in mature RBCs: (1) The anaerobic glycolysis pathway (Embden–Meyerhof pathway); (2) The pentose phosphate pathway; and (3) nucleotide metabolic pathways.

The Embden–Meyerhof pathway is the only source of energy in RBCs. Under physiological conditions, the majority of intracellular glucose is metabolized into pyruvate or lactate by the anaerobic Embden–Meyerhof pathway to generate Adenosine Triphosphate (ATP). The generated ATP is vital for RBC biological functions, including glycolysis itself, and cellular structures. For instance, ATP is essential for kinase reactions needed for cytoskeleton protein phosphorylation, which is crucial for the integrity and deformability of RBCs (Betz et al., 2009). Additionally, ATP molecules play a key role in membrane phospholipids asymmetry by controlling ATP-dependant phospholipids transporters (Daleke, 2008). In microcirculation, where O_2tension is low, RBCs release not only O_2 but also ATP, whose vasodilating effects increase perfusion flow (Sprague and Ellsworth, 2012). In addition to generating ATP, Embden–Meyerhof pathway generates nicotinamide adenine dinucleotide (NADH), an essential molecule for reducing methemoglobin to hemoglobin. A shunt within the glycolysis pathway known as the Rapoport-Luebering shunt is responsible for the formation of 2,3-bisphosphoglycerate (2,3-BPG), an important cofactor for modulating hemoglobin affinity to $O_2$for optimal O_2 delivery to tissues (van Wijk and van Solinge, 2005).

The hexose monophosphate pathway consumes approximately 10% of the available glucose, under physiological conditions, to maintain a high ratio of NADPH to NADP. NADPH is critical to convert the oxidized form of glutathione (GSSG) to the reduced form (GSH) and thus protecting critical structures like membrane lipids and cytoskeleton proteins from oxidation. Under high oxidative stress conditions, the pentose phosphate pathway consumes more glucose to compensate for the depleted GSH (McMullin, 1999). Mature RBCs lack the ability for de novo synthesis of the adenine nucleotides needed for ATP production. Instead, salvage pathways are used to recycle the existing adenosine phosphate pool (Prchal and Gregg, 2005).

Multiple enzymes control the different metabolic pathways in RBCs, some of which are key enzymes in the concerning pathway. For example, glycolytic flux is mainly controlled by hexokinase, phosphofructokinase, glucose phosphate isomerase, and PK. The hexose monophosphate shunt is controlled by glucose-6-phosphate dehydrogenase. Pyrimidine 5′ nucleotidase is critically involved in the salvage pathway for nucleotide metabolism. Inherited abnormalities in one of these enzymes, hereditary enzymopathies, may result in chronic hemolytic anemias which vary in severity according to the relative importance of the mutated enzyme with regard to the functionality or the stability of this enzyme. The most prevalent hereditary enzymopathies of RBCs result from glucose-6-phosphate dehydrogenase (G6PD) deficiency and PK deficiency (van Wijk and van Solinge, 2005).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency

G6PD deficiency is the most prevalent human enzyme deficiency, affecting hundreds of millions of people around the world. The disease is inherited as X-linked recessive trait. The majority of G6PD mutations are missense mutations in G6PD gene located on the X chromosome (band X q28). G6PD deficiency can be categorized based on the severity into three main variants: (1) Variants associated with episodic or acute hemolytic anemias; (2) Variants associated with chronic hemolytic anemias; (3) Variants associated with no clear risk of hemolysis (Gregg and Prchal, 2008).

G6PD activates the initial step of the hexose monophosphate shunt, which is needed to maintain high levels of NADPH and, consequently, GSH. Reduced glutathione is a sulfhydryl containing tripeptide acting intracellularly as a reducing agent. Reactive Oxygen species (ROS) are by-products of normal physiological intracellular reactions. However, ROS levels significantly rise upon exposure to exogenous factors like ingestion of drugs with high redox potentials, ingestion of fava beans, and during infections. Under normal conditions, oxidative damage is reversed by adequate levels of GSH. However, G6PD deficient RBCs have low levels of GSH, and they are more susceptible to damaging effects of the accumulated ROS resulting in hemolysis (Cappellini and Fiorelli, 2008). The exact molecular mechanisms of RBC hemolysis in G6PD deficiency are unclear. The intracellular accumulation of ROS may induce oxidation and clustering of membrane proteins, precipitation of oxidized hemoglobin on the inner membrane leaflet, and destabilization of the RBC membrane. Pantaleo et al. found an increase in oxidation and phosphorylation of band 3 proteins in G6PD-deficient RBCs, and that resulted in an increase in RBC hemolysis as well as an increase in the release of microvesicles containing hemichrome (Pantaleo et al., 2011).

Hemolytic episodes may stop despite the continuation of the drug administration or persistence of infection. This is probably due to the elimination of RBCs with lowest G6PD activity and, hence, lowest GSH levels. G6PD enzyme activity decreases during RBC aging; reticulocytes and younger RBCs usually have higher levels of G6PD. Red blood cell morphology may change in G6PD deficiency. Although in general RBC morphology is normal in RBC enzymopathies, peripheral blood smears of G6PD deficiency may display characteristic "bite cells" due to the oxidative denaturation of hemoglobin (Cappellini and Fiorelli, 2008).

Pyruvate kinase (PK) deficiency

Pyruvate kinase (PK) deficiency is the most common glycolytic enzyme defect and a relatively common cause of hereditary non-spherocytic hemolytic anemias. Patients have variable degrees of hemolysis, leading to mild compensated anemia, moderate hemolysis that exacerbates during infection, to severe transfusion-dependant chronic hemolytic anemia. The disease is due to missense mutations in the *PKLR* gene, resulting in generating a defective isoenzyme of PK-R, the PK isozyme which is specific to RBCs. PK deficiency is inherited in an autosomal recessive manner. PK is a key enzyme in Embden–Meyerhof glycolysis; it catalyses the transfer of a phosphoryl group from phosphoenolpyruvate to ADP to generate ATP (van Wijk and van Solinge, 2005). Although ATP molecules are crucial for the stability of the RBC membrane and for several enzymatic activities, the exact underlying mechanism of RBC hemolysis in PK deficiency is unclear. ATP deficiency is difficult to demonstrate, and other disorders accompanied with ATP depletion do not show hemolysis. Also, the selective splenic sequestration of young PK-deficient RBCs and its relationship with RBCs structure remain to be clarified (Zanella et al., 2005; Gregg and Prchal, 2008).

HEREDITARY HEMOLYTIC ANEMIAS DUE TO HEMOGLOBINOPATHIES

Hemoglobin is the main oxygen carrier in humans. It is a tetrameric protein composed of two pairs of different globin chains. Normal adult hemoglobin is mainly comprised of Hemoglobin A (Hb A), which is composed of two α-globin and two β-globin chains. Hemoglobin concentration (32–35 g per 100 mL of packed RBCs), the integrity and the solubility of these hemoglobin molecules, and their oxygen affinity are crucial factors for preserving the RBC's main function, oxygen delivery. More than 1000 mutations in globin-encoding genes have been found to affect hemoglobin synthesis, solubility, stability, or oxygen affinity. Such inherited hemoglobin anomalies may perturb the intracellular viscosity of RBCs and cell deformability, increasing the splenic sequestration of poorly deformable cells. Moreover, these perturbations may trigger proteolytic reactions, associated with ROS release, which may ultimately result in hemolytic anemias (Kuypers, 2008; Steinberg et al., 2008).

Hemoglobin anomalies can be associated with quantitative or qualitative abnormalities. Qualitative anomalies result from structurally altered hemoglobin, which has perturbed physical and/or chemical properties. Quantitative hemoglobinopathies are due to a defective production of otherwise normal globin chains. Examples of pathological conditions pertaining to the first group are sickle cell anemia, hereditary met-hemoglobinemia, high oxygen affinity polycythemia, and low oxygen affinity cyanosis. Examples of pathological conditions related to the quantitative anomalies are α- and β- thalassemias. By far the most prevalent and important conditions are sickle cell disease and the thalassemia (Poyart and Wajcman, 1996).

Sickle cell disease

Sickle cell disease is a hereditary disorder in which patients inherited a point mutation in the β-globin chain gene, encoding for valine instead of glutamic acid at position 6. With few exceptions, people with one sickle hemoglobin (HbS) gene are asymptomatic (sickle cell trait). People who have homozygosity for the mutation that causes HbS suffer from sickle cell disease. Sickle hemoglobin (HbS) polymerizes and undergoes transformation from the soluble state to a highly viscous semisolid gel under low oxygen tension conditions (Steinberg et al., 2008). An outcome of hemoglobin polymerization is the change in the morphology of RBCs, which *in vitro* become sickled, depending on the deoxygenation rate. HbS polymerization is different under *in vivo* conditions. The amount of polymer existing in the majority of cells *in vivo* is determined by the RBC hemoglobin content, the physiological oxygen tension, and the delay time for polymerization. Such factors affect the kinetics of polymerization and influence our understanding for the disease pathophysiology (Mozzarelli et al., 1987).

The clinical manifestations of sickle cell disease are downstream complications related to the formation of hyperdense RBCs (Lew and Bookchin, 2005) causing vaso occlusion. Main clinical symptoms include hemolytic anemia, recurrent painful episodes, chronic organ deterioration, particularly the spleen and kidneys, and various acute complications. However, the severity of sickle cell disease varies, and the genetics underlying this variability are far from being completely understood. One important phenotypic modifier concerns the co-inheritance of HbS with other mutant α- or β-thalassemia gene mutations (Poyart and Wajcman, 1996).

HbS polymerization cause structural damage, thereby altering the red cell membrane. In addition, unstable HbS may cause oxidative damage. Red blood cell membrane damages are associated with PS externalization and band 3 clustering, which may promote RBC opsonization, and consequently, RBC phagocytosis (Waugh et al., 1986; de Jong et al., 2001). Additionally, these membrane alterations may induce membrane surface area loss through releasing microvesicles. The release of microvesicles from sickled RBCs was first described by Allan et al. They reported that reoxygenation of sickled RBCs could result in the release of spectrin free- and polymerized hemoglobin-containing microvesicles (Allan et al., 1982). Others reported high levels of circulating microvesicles in patients with sickle cell anemia (Westerman et al., 2008).

Thalassemias

Thalassemias are a heterogeneous group of hereditary anemias which result from reduced biosynthesis of one of the two globin chains, α- or β- globin chain, that are needed to form the adult hemoglobin tetramer, HbA. Individual disorders of thalassemias are named according to the affected globin chain. For instance, patients with α-thalassemia have absent or reduced α- globin chains while patients with β-thalassemia have absent or reduced β-globin chains (Steinberg et al., 2008). The α- and β-globin chains in thalassemias are usually of normal structure.

Pathophysiological consequences of thalassemias arise from the underproduction of hemoglobin and the intracellular accumulation of excess globin subunits. The underproduction of hemoglobin diminishes oxygen carrying capacity of surviving RBCs. More importantly, the excess globin subunits probably precipitate on the membrane inner leaflet, inducing damages in the membrane cytoskeleton and decreasing the RBC deformability. Moreover, the rapid degradation of free globin chains in RBC precursors may result in destruction of RBC precursors in bone marrow associated with ineffective erythropoiesis (Poyart and Wajcman, 1996; Steinberg et al., 2008).

THE LINKAGE BETWEEN HEREDITARY HEMOLYTIC ANEMIAS AND RED BLOOD CELL-DERIVED MICROVESICLES

The relationship between hereditary hemolytic anemias and RBC vesiculation is far from being completely deciphered. The diverse molecular pathologies of hereditary hemolytic anemias probably play a key role in determining RBC deformability, fragility and vesiculation. Thus, the phenotype and the levels of the circulating RBC-derived microvesicles of each disorder are primarily determined by the underlying molecular defects. Moreover, therapeutic interventions, like splenectomy and pharmaceutical compounds, may differently modify RBC vesiculation for each disorder (Bütikofer et al., 1989; Westerman et al., 2008).

Red blood cell deformability probably shows a negative correlation with RBC vesiculation. Such a correlation can be demonstrated by a reduction in the extent of RBC deformability, which is paralleled with an increase in microvesicle levels during blood storage (Almizraq et al., 2013). However, others found that there is a minimal change in RBC deformability during blood storage, and such a finding could be explained by the parallel decline in MCHC, which may compensate the effect of surface area loss on RBC deformability (Cluitmans et al., 2012). In hereditary membranopathies, like HS and HE, the instability of the RBC membrane is most likely accompanied by a consequent membrane loss through vesiculation, generating less deformable RBCs which have less surface/volume ratio. By using a quantitative flow cytometry method, Mullier et al. found that HS patients' blood contained higher levels of RBC-derived microvesicles compared to healthy controls (Mullier et al., 2012). Regarding hereditary hemoglobinopathies, unstable hemoglobin induces oxidation of membrane proteins and lipids, deposition of hemoglobin on the membrane, and changes in the intracellular viscosity, and that all results in poorly deformable RBCs (Mokken et al., 1992). Red blood cell tendency to shed microvesicles may reflect separation of the membrane bilayer from the underlying skeleton by spicules of polymerized deposited hemoglobin. For instance, in sickle cell disease, a substantial percentage of cell-free hemoglobin is encapsulated in RBC-derived microvesicles released during sickling. Concerning hereditary RBC enzymopathies, the correlation between RBC deformability and vesiculation is less clear than other hereditary hemolytic disorders, probably due to two main reasons; the genetic diversity of enzymopathies and the dependency of membrane damage on the level of oxidative stress. Johnson et al. reported increased deformability of G6PD-deficient RBCs, which may be explained by the fact that RBCs in many cases of glycolytic enzyme deficiency are foetal-like RBCs, which are more deformable (Johnson et al., 1999). However, others reported that G6PD-deficient RBCs were particularly susceptible to oxidative stress-induced damage, and that results in a dramatic reduction in RBC deformability (Gurbuz et al., 2004). Notably, it is likely that patients may have different subpopulations of RBCs, with different deformability, and that affects the overall deformability results (Mokken et al., 1992). The increased susceptibility of RBCs to oxidative damage in hereditary enzymopathies may cause microvesicle shedding. G6PD-deficient subjects may have high levels of circulating RBC- and platelet-derived microvesicles when compared to healthy controls, and microvesicle concentration probably has a negative correlation with G6PD activity (Nantakomol et al., 2012). However, vesiculation in other hereditary enzymopathies remains to be understood.

Treatment modalities of hemolytic anemia, like splenectomy and pharmaceutical compounds, may modify RBC deformability and vesiculation. The anatomic structure of the spleen is challenging for RBC metabolic machinery and deformability. RBCs have to go through repeated deformations when passing through both the capillary bed and the inter-endothelial slits of the spleen, where RBCs are concentrated and their intracellular

metabolism is stressed. Patients with hereditary hemolytic anemias are likely to exhibit RBCs with morphological abnormalities or metabolically distressed RBCs, which cannot withstand such a splenic challenge. Thus, these abnormal RBCs are likely to be sequestered by the spleen and subsequently phagocytosed by splenic macrophages. Such splenic sequestration and erythrophagocytosis are responsible for the development of anemia and splenomegaly (Mebius and Kraal, 2005). Notably, in sickle cell disease the spleen is also one of the affected organs. During the first decade of life the spleen is usually enlarged but due to vaso occlusion events and infarction it undergoes progressive atrophy leading to autosplenectomy (Pearson et al., 1979, 1985; Al Salem, 2011).

Splenectomy is indicated as a last option to treat the progressive increase in blood transfusion requirements due to the splenomegaly-mediated hemolysis. In many cases of hereditary hemolytic anemias, splenectomy may improve anemia, reduce the rate of hemolysis, and eliminate symptoms of splenomegaly (Reliene et al., 2002; Casale and Perrotta, 2011). However, splenectomy is not a curative intervention, and sometimes contraindicated due to post-surgery complications. Although splenectomy may be beneficial for patients with HS or sickle cell disease, it is contra-indicated for patients with HSt, who may develop a hypercoagulable state after splenectomy resulting in thrombotic episodes or pulmonary hypertension (Stewart et al., 1996; Stewart and Turner, 1999). Also splenectomised β-thalassemia intermedia patients may develop venous thromboembolic complications like pulmonary embolism and deep venous thrombosis (Westerman et al., 2008). The underlying mechanisms of these thromboembolic events are not clear, and the role of RBCs-derived microvesicles in these events remains ill-defined. The transmembrane asymmetry of phospholipids and PS externalization may be different among the different hereditary hemolytic anemia, and these differences may play a crucial role in the hypercoagulable state. HS and HE RBCs were shown to have a normal transbilayer phospholipid asymmetry, whereas RBCs of some stomatocytosis variants were shown to have altered phospholipids asymmetry in addition to increased adherence to endothelial cells (de Jong et al., 1999; Gallagher et al., 2003). It is likely that splenectomy in stomatocytosis prolongs the circulation of the PS-exposing RBCs, developing a hypercoagulable state. Westerman et al. demonstrated that splenectomy may differently affect the levels of PS-exposing microvesicles between sickle cell anemia and thalassemia intermedia (Westerman et al., 2008). In patients with thalassaemia intermedia, splenectomy was shown to increase the levels of PS-exposing microvesicles, potentially due to the diminished clearance of abnormal RBCs, which produce these PS-exposing microvesicles. Although vesiculation may be facilitated by the spleen in healthy subjects, this would not explain the post-splenectomy increase in RBC-derived microvesicles in thalassaemia intermedia patients (Willekens et al., 2003; Westerman et al., 2008).

The use of medications for therapeutic needs should be carefully considered in patients with hereditary hemolytic anemias. Also, the diagnosis of such diseases and the analysis of RBCs-derived microvesicles in hemolytic anemias should be carefully performed with respect to the patient's medical history. Exposure of RBCs to pharmacologically active compounds, which have different physicochemical properties and mechanisms of actions, may have different impacts on RBC morphology, deformability and vesiculation. For instance, many drugs are amphiphilic in nature, meaning that that they have hydrophobic and polar parts.

Based on the bilayer couple hypothesis, which hypothesizes that RBC outer and inner leaflets respond differently to perturbations, the morphological transformations of RBCs, and the consecutive vesiculation, can be predicted from the nature of the drugs interacting with RBCs (Sheetz and Singer, 1974). For instance, anionic drugs, like indomethacine and phenyibutazone, preferentially accumulate in the RBC outer leaflet and induce RBC spiculation (echinocytosis) which results in membrane loss through vesiculation. Such an interaction is electrostatically more favorable due to the net negative charge of the inner membrane leaflet. On the other hand, cationic drugs, like chlorpromazine, primaquine and tetracaine, preferentially accumulate in the inner leaflet and they induce a stomatocytic transformation of RBC morphology, resulting in inhibition of vesiculation and/or endovesiculation (Bütikofer et al., 1987; Schreier et al., 2000). It is not clear if the physical expansion of the inner or the outer leaflet of the RBC membrane, based on the preferential accumulation of amphiphilic drugs, is the sole event that affects RBC transformations and vesiculation. Red blood cells possess membrane-associated enzymes, which have different topological arrangements/distribution across the cell membrane. For examples, ATPases, proteinases and acetylcholinesterase are known to be confined to the membrane, whereas enzymes of glutathione and glucose metabolism are found in the cytosol and associated with the inner leaflet of the membrane (Schrier, 1977). Some of these enzymes are involved in regulating RBC morphology, trafficking of cations, and cell membrane rigidity. In addition to the physical impacts, the intercalation of amphiphilic drugs may differently affect the membrane-associated enzymes based on which membrane leaflet is affected, and that may result in vectorial or one-sided changes in the membrane enzymatic activities, leading to differences in the RBC vesiculation behavior (Schrier, 1977; Bütikofer et al., 1989; Schreier et al., 2000). Additionally, the effects of amphiphilic drugs on RBC deformability and vesiculation can be explained by the possible mechanisms of action of antimicrobial peptides. An antimicrobial peptide, like gramicidin D, has amphiphilic nature, and it may affect the cell membrane integrity and ion permeability by two main possible mechanisms: (1) the carpet-like mechanism; and (2) the barrel-stave mechanism. In the first mechanism, the peptide acts as a detergent by lining on the cell membrane surface till reaching a critical concentration at which the lipid bilayer is disrupted. In the latter mechanism, a membrane channel is formed, in which the hydrophobic α- helical or β-sheets of the peptide interact with the acyl chains of the membrane whereas the hydrophilic portion lines the interior of the channel. Such a channel disrupts the osmotic state of the cell by disrupting membrane permeability (Schreier et al., 2000; Yeaman and Yount, 2003). Interestingly, beside amphiphilic drugs, other pharmaceutical molecules seem to intercalate with the RBC outer leaflet. For instance, Baerlocher et al. found that Fluorouracil (5-FU), an anti-cancer drug, could preferentially expand the outer leaflet of RBCs, inducing

cell echinocytosis and shedding PS-exposing microvesicles. Such microvesicles are potent stimulators of coagulation and may be responsible for the thromboembolic complications that occur at high therapeutic doses of 5-FU (Baerlocher et al., 1997). However, others reported that the effects of 5-FU on RBCs may partially be explained by the depletion of RBC ATP (Spasojevic et al., 2005). Additionally, molecules that modulate calcium trafficking may be of a therapeutic value for conditions associated with RBCs of reduced deformability. Intracellular calcium plays crucial roles in controlling membrane deformability and vesiculation. An example of intracellular calcium modulators is zinc. Zinc therapy in sickle cell anemia may reduce RBC sickling and improve cellular deformability, potentially through inhibition of calmodulin, an intracellular mediator of Calcium activities (Brewer et al., 1977). Another example of a calcium antagonist is magnesium, which may be administered intravenously to reduce the risk of seizures in pregnant women with preeclampsia. Magnesium may partially improve the impaired microcirculation in preeclampsia by increasing RBC deformability through antagonizing the stimulation of calcium pumps and reducing ATP depletion (Schauf et al., 2004). An additional example is dipyridamole, an inhibitor of platelet activation, which may enhance RBC deformability by enhancing glucose uptake and boosting glycolysis, increasing intracellular levels of ATP, and thus maintaining a more efficient calcium pumping to the extracellular milieu (Sowemimo-Coker et al., 1983; Saniabadi et al., 1992).

INTEGRATED BIO-ANALYTICAL FINDINGS: A NEED FOR PROFILING RED BLOOD CELLS AND RED BLOOD CELL-DERIVED MICROVESICLES

Beside blood cell indices, current methodologies that are needed for the basic understanding and clinical diagnosis of hereditary hemolytic anemias encompass genetic analysis, protein analysis, assessment of RBC deformability, flow cytometry, assays to rule out immune-hemolytic anemia, and enzyme activity assays. Rarity and diversity of hereditary hemolytic anemias, and our incomplete understanding of RBC-derived microvesicles, which can be considered as miniatures of the aberrant RBCs, represent a challenge for the scientific community. Overcoming such a challenge is needed to have a better comprehension of the diseases and to develop new therapeutics for each disease. An approach to overcome these challenges is to combine and integrate data from the several experimental platforms on RBCs and their microvesicles to provide new insights into the molecular interactions, cellular and vesicular (sub) phenotypes, and disease processes. Such profiling of hereditary hemolytic anemias can be started by integrating data generated by robust methods like ektacytometry, flow cytometry, nanoparticle characterization and proteomics.

Cytometry

Ektacytometry is defined in the dictionary of cell and molecular biology as a "Method in which cells (usually RBCs) are exposed to increasing shear-stress and the laser diffraction pattern through the suspension is recorded; it goes from circular to elliptical as shear increases. From these measurements, a deformability index for the cells can be derived." (Lackie, 2007). Determination of deformability index (DI), also known as elongation index (EI), is indicative to cell membrane rigidity. A variety of ektacytometry is the osmotic gradient ektacytometry, in which RBCs are suspended in solutions of polyvinylpyrrolidone (PVP) of varied tonicity and the DI is determined as a continuous function of the suspending medium osmolality at a constant shear rate. By keeping in mind that RBC membrane is highly permeable to water, any change in the osmolality of the suspension medium results in a change in the hydration state of RBCs (Johnson and Ravindranath, 1996). Red blood cell deformability is determined by RBC internal viscosity, membrane surface to volume ratio, and membrane rigidity. Alterations of one or more of these three determinants can be detected by interpreting changes in the osmotic gradient deformability profile. Since the underlying molecular defects of hereditary hemolytic anemias are different, a number of these anemias can be rapidly diagnosed by their distinctive osmotic gradient deformability profile (Clark et al., 1983; Johnson and Ravindranath, 1996; Da Costa et al., 2013). Combining these profiles with other haemorheological parameters like the aggregation behavior of RBCs may be of added value in further profiling of hereditary hemolytic anemias. An example of such a hybrid technique is the commercially available instrument called LORCA (Laser-assisted Optical Rotational Cell Analyzer) (Hardeman et al., 2001).

Flow cytometry is a simple, reproducible, and high throughput method that can be used for qualitative and quantitative immunophenotyping of RBCs. The availability of a wide array of fluorescently labeled antibodies can be utilized to detect several RBC surface and cytoplasmic antigens that may vary among hereditary hemolytic anemias. Additionally, laser scattering at different angles can distinguish differences in cell size and internal complexity (Brown and Wittwer, 2000; Davis, 2001). An automated hematology analyser like the Abbott cell-dyn Sapphire can also be modified to detect morphological abnormalities of the analyzed RBCs (Kim et al., 2003).

Nanoparticle characterization

The submicron size of microvesicles poses several challenges for analysis. To characterize their biological characteristics, such as surface proteins, tools that have originally been developed for cells are usually employed, like flow cytometry. The limited scattering properties of nanosized particles make flow cytometry of microvesicles not straightforward.

This is primarily caused by the lack of standardized assay protocols in addition to the heterogeneity in size (where the majority of the population usually is well below the detection threshold) and composition of microvesicles (Shah et al., 2008; Sustar et al., 2011). Another challenging factor is that the clearance of RBCs-microvesicles seems to be a very efficient process (Willekens et al., 2005). Thus, it is not clear if the flow cytometry quantification of these microvesicles represent their constant circulating levels *in vivo* or that these levels are highly variable in time. Thus, there is a need to standardize flow cytometry protocols and to profile the phenotypes of RBCs and their microvesicles in the different hemolytic anemias.

There are two main approaches that may be employed in analysing RBC-derived microvesicles: (1) selection of microvesicles based on a size criterion followed by analysis of

the fluorescent signals of events limited to this preselected size, or (2) initial selection is based on the fluorescent signals then limiting the analysis by scatter gating to these signals (Xiong et al., 2012). Among the generally investigated antigens of RBCs and RBC-derived microvesicles are glycophorin A, PS, and band 3. Glycophorin A is expressed on the vast majority of RBC-derived microvesicles, and it is considered as a universal marker to distinguish RBC-derived microvesicles from those of other cell lineages (Pattanapanyasat et al., 2004). Vesicle PS can be labeled by Annexing V. However, it is not clear if all RBCs-derived microvesicles expose PS (Barteneva et al., 2013). Flow cytometry can also be used to quantify the transmembrane band 3 proteins by labeling with the dye eosin-5′ maleimide (EMA). EMA staining is frequently used in the diagnosis of HS (Girodon et al., 2008). As aforementioned, the molecular defects in HS are heterogeneous; a common feature of HS RBCs appears to be a weakening of the tethering points associated with band 3, and that facilitates RBC vesiculation and losing band 3 from the RBC membrane into the released microvesicle. Indeed, Stoya et al. and Knowles et al. reported that the EMA fluorescence signal was reduced in HS RBCs and after heat- or shear-induced vesiculation of normal RBCs, and the released microvesicles showed membrane associated-EMA labeled band 3 proteins (Knowles et al., 1997; Stoya et al., 1997). Although the EMA test is highly sensitive, it is not without drawbacks. EMA binds covalently to the Lys430 on the extracellular loop of band 3 protein but it may also bind to sulfhydryl groups expressed by Rh, RhAg and CD47. Moreover, some mutants of band 3 fail to bind to the dye EMA. The use of EMA test is also less effective in diagnosis of HS due to ankyrin defects (Girodon et al., 2008).

From the pharmaceutical technology field a number of techniques are available to characterize physicochemical properties of vesicles, such as size and surface charge. These can be measured by a variety of techniques such as dynamic light scattering, electron microscopy, tunable resistive pulse sensing, or nanoparticle tracking analysis. These techniques, however, have difficulties in assessing the biological heterogeneity of samples. As an example, nanoparticle tracking analysis studies the brownian motion of microvesicles, which can be tracked through the scattered light after laser illumination. Since the antigen number on the surface of nanosized vesicles is relatively small, it is difficult to extract biological information on surface markers as the absolute signal remains low.

The proteome of red blood cells and their microvesicles

Mature RBCs are anucleate cells with few intracellular structures and no capability to synthesize new proteins. Thus, their proteome is less complex compared to other cell types. This fact, along with the comprehension of RBC physiology, opens up an opportunity to use proteomic techniques for a better understanding of hereditary hemolytic anemias. Mass spectrometry (MS) is a powerful, automated and high throughput tool, which enables us to analyse the proteome of RBCs in health and disease. Such a comparative proteomic analysis can provide us with considerable insights into disease-related alterations and severity, potential diagnostic markers, potential therapeutic targets, and drug-induced changes (Prudent et al., 2011). Such studies may improve our understanding of the underlying complex pathophysiology of hereditary hemolytic anemias like sickle cell disease, HS and HE (Kakhniashvili et al., 2005; Margetis et al., 2007; Pasini et al., 2010; Yuditskaya et al., 2010). Despite advances in MS, proteomic studies of RBCs in hereditary hemolytic anemias are limited.

There is a growing interest in using proteomic technologies for high throughput protein profiling of extracellular vesicles. Such an interest is driven by new findings pointing to the crucial roles in intercellular communications and other pathophysiological processes. With respect to RBC-derived microvesicles, little is known about their protein cargo (Simpson et al., 2009). Proteomic data are mainly available on microvesicles derived from normal RBCs during storage under blood bank conditions (Tissot et al., 2013). Bosman et al., in a series of proteomic studies, investigated the effect of storage conditions and RBC ageing on RBC vesiculation (Bosman et al., 2008, 2010, 2012; Willekens et al., 2008). By comparing the proteome of RBC-derived microvesicles with the proteome of the parental RBC membrane, the Bosman group hypothesized that RBCs-derived microvesicles and vesiculation may be a means to selectively remove senescent cell antigens and other molecules to postpone the premature elimination of RBCs from the circulation. In the context of hereditary hemolytic anemias, Chaichompoo et al. showed that the levels of 29 proteins of microvesicles derived from β-thalassemia/hemoglobinE patients were significantly altered when compared to controls. These proteins included peroxiredoxin 6, apolipoprotein E, cyclophilin A and heat shock protein 90, and they were primarily involved in the regulation of RBC redox reactions, phospholipid turnover, and blood coagulation (Chaichompoo et al., 2012).

CONCLUDING REMARKS

Red blood cells-derived microvesicles, once thought of as cell debris, now seem to have important roles in regulating blood homeostasis and modulating immune response and other pathophysiological processes. Red blood cell microvesiculation is a tightly controlled process and sorting of bioactive molecules into microvesicles occurs in a selective manner. The molecular defects of hereditary hemolytic anemia are diverse, and they potentially affect RBC vesiculation. Microvesicles released in the different hereditary hemolytic anemias are potentially unequal and may have different biological effects. As the exact role of red cell derived miscrovesicles in many of the hemolytic anemias is currently unknown and unexplored this opens up a whole area of exciting research in the coming period of time. Understanding the molecular bases of hereditary hemolytic anemias, and their impact on RBCs deformability and microvesiculation, can provide new insights into the pathophysiology of these anemias and may lead to the discovery of new diagnostic markers and therapeutic molecules.

AUTHOR CONTRIBUTIONS

Amr Alaarg, Raymond M. Schiffelers, Wouter W. van Solinge, and Richard van Wijk wrote the manuscript. All authors approved the final version of this manuscript.

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Survival of red blood cells after transfusion: processes and consequences

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The currently available data suggest that efforts toward improving the quality of red blood cell (RBC) blood bank products should concentrate on: (1) preventing the removal of a considerable fraction of the transfused RBCs that takes place within the first hours after transfusion; (2) minimizing the interaction of the transfused RBCs with the patient's immune system. These issues are important in reducing the number and extent of the damaging side effects of transfusions, such as generation of alloantibodies and autoantibodies and iron accumulation, especially in transfusion-dependent patients. Thus, it becomes important for blood bank research not only to assess the classical RBC parameters for quality control during storage, but even more so to identify the parameters that predict RBC survival, function and behavior in the patient after transfusion. These parameters are likely to result from elucidation of the mechanisms that underly physiological RBC aging *in vivo*, and that lead to the generation of senescent cell antigens and the accumulation of damaged molecules in vesicles. Also, study of RBC pathology-related mechanisms, such as encountered in various hemoglobinopathies and membranopathies, may help to elucidate the mechanisms underlying a storage-associated increase in susceptibility to physiological stress conditions. Recent data indicate that a combination of new approaches *in vitro* to mimick RBC behavior *in vivo*, the growing knowledge of the signaling networks that regulate RBC structure and function, and the rapidly expanding set of proteomic and metabolomic data, will be instrumental to identify the storage-associated processes that control RBC survival after transfusion.

Keywords: aging, autoimmunity, erythrocyte, membrane, transfusion, vesicle

INTRODUCTION

The final days of the erythrocyte's life are characterized by the appearance of an aging-specific removal signal. The signal is a neoantigen that is derived from the integral membrane protein band 3, and the removal is phagocytosis that is initiated by binding of autologous IgG and subsequent recognition by macrophages in the liver and possibly in the spleen (Kay, 1975; Safeukui et al., 2012). Since the seminal papers of Kay and coworkers (Kay, 1975, 1978, 1981, 1984; Kay and Bennett, 1982; Kay et al., 1983, 1988a; Safeukui et al., 2012) with the observations and experiments supporting and/or based on this concept, recognition of old erythrocytes by a physiological autoimmune reaction has been generally accepted as responsible for the physiological disappearance of erythrocytes after 120 days in the circulation (Clark, 1988). However, little progress has been made with respect to the identity of the molecular mechanism(s) leading to the formation of the aging or senescent cell-specific antigen. This is the more frustrating, since the erythrocyte has the potential of becoming a model for cellular aging, as it once was for membrane structure. Also, the experimental and conceptual framework that has arisen around this concept, has the potential of becoming instrumental in the generation of biomarkers of various diseases, and in the expansion of our knowledge of the regulation of cell morphology and metabolism.

The aging framework is commonly—and often implicitly—used to interpret the molecular events occurring during erythrocyte storage in blood bank conditions (Bosman et al., 2008a, 2010a). The link between aging *in vivo* and aging *in vitro* is extended by the study of hereditary anemias caused by increased erythrocyte removal. The resulting triangle has been given at least one extra dimension by recent data indicating that, at the current scientific and technical level, the truly relevant blood bank conditions are not those that determine erythrocyte survival in the blood bank, but those that affect function and survival after transfusion (Bosman et al., 2011). Yet another, new dimension is formed by the status of the erythrocyte-receiving patient, e.g., the activity of the immune system and/or the spleen, as an effector of the survival of the transfused erythrocytes (Gould et al., 2007; Dinkla et al., 2012a,b).

The present review starts with a summary of the currently available knowledge of the molecular structure, function and metabolism of the aging erythrocyte in the healthy individual. This summary is the starting point for a review of the data obtained *in vitro* and from patients with hereditary erythrocyte pathologies, based on the view that this may help to deduce the most likely molecular mechanism(s) leading to the aged phenotype. The resulting synthesis constitutes the framework for a discussion of the storage lesions, focussing on their impact on the survival of erythrocytes after transfusion.

CHARACTERISTICS OF ERYTHROCYTE AGING *in vivo*

An overview on the available data, strictly limited to those obtained by analysis of erythrocyte aging *in vivo* in healthy people, shows the following:

LIFESPAN

The maximal lifespan of erythrocytes is 120 days, with a rather small variation of approximately 10 percent. This variation may be due to variations in methodology, such as the analysis of appearance and disappearance of metabolic labels from the circulation, and of the disappearance of erythrocytes labeled with various markers after autologous transfusion, or after transfusion of erythrocytes with differences in minor blood groups (Werre et al., 2004; Bosman et al., 2012a). Alternatively, the variability in maximal lifespan may also be due to inter-individual variations in erythrocyte homeostasis, as has become apparent especially in recent blood bank donor research (Wenk et al., 2011; Dinkla et al., 2013). The relatively small variation in lifespan observed in all studies, however, suggests a gradual, multi-step mechanism rather than a random, disastrous insult, as well as a very efficient removal process.

VOLUME AND DENSITY

With increasing time in the circulation, erythrocytes become smaller and more dense. A detailed analysis of these changes shows that, with age, erythrocytes loose 30% of their volume and 15–20% of their hemoglobin, whereas the hemoglobin concentration increases by 14%. This implies that, with age, erythrocytes lose proportionally more water than hemoglobin. Because the decrease in volume is larger than the decrease in surface area, the surface to volume ratio increases. This theoretically positive effect on deformability is abolished by the increase in the hemoglobin concentration, and probably by a decrease in the membrane elasticity (Bosch et al., 1994). Using the percentage of glycated hemoglobin, HbA1c, as a marker of cell age in combination with cohort labeling, survival studies and hemocytometry, Werre and coworkers (Van der Vegt et al., 1985; Bosch et al., 1992, 1994) established that age-related purification of cell fractions on the basis of density alone has inherent restrictions. A similar conclusion can be drawn using another cell age marker, the 4.1a:4.1b ratio, which increases as the result of non-enzymatic deamidation (Mueller et al., 1987; Lutz et al., 1992; Ciana et al., 2004). The lighter fractions are strongly enriched for reticulocytes and young erythrocytes, but the dense fractions are much more heterogeneous with respect to cell volume and cell age. When counterflow centrifugation is followed by density centrifugation, the mean corpuscular volume decreases from 101 fl in the fraction containing the lightest and largest erythrocytes to 72 fl in the fraction with the most dense and smallest cells (Bosch et al., 1992). Combining the two separation techniques results in a considerable reduction in the cell volume-based distribution curves, an almost complete absence of overlap in the erythrocytograms of the lighter-larger and the denser-smaller fractions, and the largest difference in the percentage HbA1c between these fractions (Bosch et al., 1992). Thus, a combination of separation techniques based on volume and density yields erythrocyte fractions with a greater difference in mean cell age than does separation on the basis of density or volume alone.

VESICULATION

Based on observations in splenectomized individuals and on the analysis of subcellular blood fractions, it has been postulated that, under normal conditions, vesiculation is responsible for the aging-associated loss of hemoglobin. The striking resemblance between the hemoglobin composition of blood-borne vesicles and that of old erythrocytes, supports the conclusion that there is a continuous loss of hemoglobin in vesicles, which accelerates during the second half of the erythrocyte lifespan (Willekens et al., 2003). In the oldest erythrocytes of asplenic individuals, the decrease in hemoglobin is absent, concomitant with an increase in the absolute amounts of glycated and otherwise modified hemoglobin species (Willekens et al., 2003). Together with the previous observations that erythrocytes of patients without a functional spleen have an increased number of hemoglobin-containing vacuoles (Reinhart and Chien, 1988), and that there is a positive relation between the vacuole-containing erythrocytes and the percentage HbA1c (De Haan et al., 1988), these data suggest that hemoglobin-containing vesicles within old erythrocytes are removed from the erythrocytes in the spleen. However, it is not likely that vesiculation and vesicle removal occur only in the spleen. A rough calculation based on the vesicle concentrations measured in the blood (Willekens et al., 2008), combined with the kinetics of vesicle disappearance as measured in a rat model (Willekens et al., 2005), indicates that erythrocyte-derived vesicles are phagocytized almost directly after they are generated, even before they can reach the venous circulation. Immunological, biochemical and proteomic analysis of the vesicles generated *in vivo* suggests that their origin and removal are intimately interwoven with the erythrocyte aging process, and especially with breakdown of band 3 (Willekens et al., 2008; Bosman et al., 2008a, 2012b). The increase in erythrocyte-derived vesicles described in various pathological conditions, most of which are directly related to erythrocyte-specific abnormalities in hemoglobin or membrane proteins, suggests a disturbed aging process in these diseases (Kozuma et al., 2011; Mahfoudhi et al., 2012). However, it has become clear that vesicles may be generated by various mechanisms, emphasizing the need for extensive analysis of these vesicles and comparison with those generated in healthy individuals (Willekens et al., 2005, 2008; Bosman et al., 2010a, 2012b; Kozuma et al., 2011; Xiong et al., 2011; Mahfoudhi et al., 2012; Nantokomol et al., 2012).

REMOVAL SIGNALS

Alterations in band 3, as indicated by the presence of breakdown products and/or band 3-containing high-molecular-weight complexes, constitute the most consistent finding in the membranes of old erythrocytes (Kay, 2005; Pantaleo et al., 2008; Willekens et al., 2008; Bosman et al., 2010a,b). Together with the specificity of the senescent cell-specific IgG, this implies a band 3-derived antigen as the main factor responsible for the removal of old erythrocytes from the circulation. There is no convincing evidence for the involvement of phosphatidylserine (PS) in physiological removal of healthy, aged erythrocytes (Willekens et al., 2008; Franco et al.,

2013). Recently, however, data have been presented indicating the involvement of the "self" antigen CD47 in phagocytosis of old erythrocytes (Burger et al., 2012). The observations that vesicles are enriched in IgG and band 3 breakdown products, and that most of them expose PS at their surface, have led to the theory that vesiculation serves to dispose damaged membrane patches (Willekens et al., 2008; Tissot et al., 2010). This mechanism would postpone the elimination of functional erythrocytes, and the fast removal of vesicles may prevent uncontrolled coagulation and inflammation (Xiong et al., 2011; Mahfoudhi et al., 2012). The current state of knowledge as described here is schematically depicted in **Figure 1**.

MECHANISMS OF ERYTHROCYTE AGING *in vivo*

So far, the data of the last decade have mostly confirmed and extended, but not deepened the picture sketched in the preceding paragraphs (Kay, 2005; Pantaleo et al., 2008; Franco et al., 2013). Incorporation of the vesicle characteristics into the aging process supports the putative early involvement of hemoglobin, and the central role of band 3 in the aging process (Salzer et al., 2008; Willekens et al., 2008; Tissot et al., 2010; Bosman et al., 2012b). The cytoplasmic domain of band 3 is a central mediator of the concentration of ATP, 2,3-DPG, NADH and NADPH (Messana et al., 1996; Chu et al., 2008; Rogers et al., 2009; Dzik, 2011). Thereby, aging-associated changes in band 3 connect changes in cell morphology and volume, deformability, and interaction between cytoskeleton and lipid bilayer with changes in the activity of the glycolytic and the pentose phosphate pathways, and possibly ion transport and release of ATP and NO as well. Binding of oxidatively modified hemoglobin, the so-called hemichromes, to the cytoplasmic domain of band 3 is likely to alter its conformation, and may thereby induce aggregation and/or increase its susceptibility to proteases. An aging-related increase in oxidation of membrane lipids and proteins may catalyze this process. Indeed, recent proteomic data show an aging-associated membrane recruitment of chaperone proteins, indicating denaturation and exposure of hitherto hidden protein domains (Bosman et al., 2012b). Also, metabolomic and biochemical data suggest a decrease in anti-oxidation defense, together with a decrease of glycolytic activity in erythrocytes aged *in vivo* (Bosman and Kay, 1988; Ghashghaeinia et al., 2012; D'Alessandro et al., 2013a). Alterations in the cytoplasmic domain of band 3 also affect binding of ankyrin, and loss of anchorage at this junction of the spectrin cytoskeleton to the lipid bilayer is the most likely cause of the formation of vesicles observed *in vivo*, and explains much of their protein composition (Sens and Gov, 2007; Gov et al., 2009). Erythrocyte aging in humans can be studied experimentally only *in vitro*, and physiological erythrocyte removal in mice is likely to differ too much from that in humans to expect that mouse studies will lead to new, relevant developments in this area (Khandelwal and Saxena, 2008). Therefore, support for the mechanisms that have been deduced from theory-driven or inventory-based comparisons of erythrocytes of various ages, has been sought in various erythrocyte-centered diseases. Detailed analysis of hemoglobinopathies, such as sickle cell disease and hemoglobin Köln, supports the relationship between hemoglobin deposition at the membrane, immunoglobulin binding, and also vesiculation (Kay et al., 1988b; Westerman et al., 2008). Immunoblot analysis of HbS-containing erythrocytes show sickling-associated and aged cell-like band 3 patterns (Bosman, 2004).

Altered vesiculation may underly the aberrant erythrocyte morphology caused by genetic abnormalities in band 3, ankyrin or spectrin leading to elliptocytosis. The role of the spleen in vesiculation is confirmed by the beneficial effect of splenectomy in these patients (An and Mohandas, 2008). It is noteworthy that the elliptocytosis-associated mutations are all located in the membrane domain of band 3, illustrating the complexity of the processes involved. This is supported by the observation that

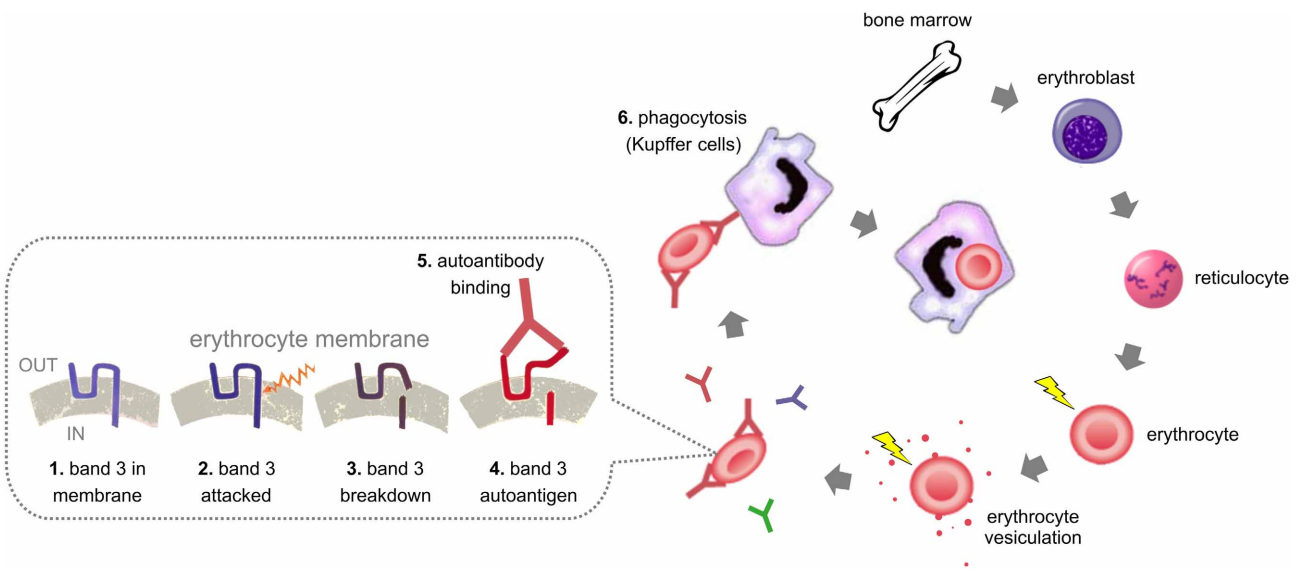

FIGURE 1 | The life and death of the erythrocyte. The numbers refer to the putative steps in the generation and recognition of removal signals.

band 3 mutations may also be associated with ovalocytosis or with stomatocytosis (Delaunay, 2007). Since the main trigger for erythrocyte loss in G6PD-deficient erythrocytes is oxidative stress (Beutler, 2008), erythrocytes of affected individuals have been used to investigate the role of oxidation in aging-associated changes in band 3. The results support a connection between hemoglobin denaturation, vesicle formation, oxidation and phosphorylation of band 3 (Minetti et al., 1996; Pantaleo et al., 2011). The latter is in line with the recent awareness of the activity in the erythrocyte of multiple signaling pathways that regulate the interaction of membrane proteins with eachother and with cytosolic proteins. Especially research of the mechanisms that regulate recruitment and activity of kinases and phosphatases is likely to reveal more insight into the processes underlying maintenance and loss of erythrocyte morphology and metabolism in health, disease and aging.

Over the years, data from comparing young with old, and control with pathological erythrocytes, have provided the tools not only to investigate erythrocyte aging in disease, but also to test theories on aging mechanisms *in vitro* (Bosman and Kay, 1988). The latter has led to two main conclusions: 1, investigation of any aging-associated susceptibility to physiological stress-mimicking conditions may reveal the most relevant lesions at the molecular level, and the mechanisms leading to these lesions (Ghashghaeinia et al., 2012); 2, the experimental treatment that results in the highest degree of similarity in morphological, structural, and functional changes to those observed during aging *in vivo*, is storage in blood bank conditions (Bosman and Kay, 1988). Oxidation *in vitro* or reduced protection against oxidative damage *in vitro* and *in vivo* are second best (Bosman and Kay, 1988; Kay et al., 1988a; Burger et al., 2013).

BLOOD BANK STORAGE, AGING, AND REMOVAL AFTER TRANSFUSION

There are many excellent articles and reviews on what happens with the erythrocyte during its sojourn in the blood bank (Hess, 2012). Here we summarize and discuss these events in relation to the aging process *in vivo*, and to their possible effect on their survival after transfusion.

LIFESPAN

Erythrocyte survival and lifespan measurements obtained with transfused erythrocytes are in good accordance with those obtained using metabolic labeling studies, indicating an overall good survival with a maximum of 135 days after transfusion (Mollison et al., 1987; Luten et al., 2004). However, almost all studies show the disappearance of 5–10% within the first 24 h after transfusion, which is followed by a linear disappearance curve. This percentage of rapidly disappearing erythrocytes increases with storage time to 25% or more (Luten et al., 2008a). These data suggest that, during their stay in the blood bank, erythrocytes become increasingly vulnerable to as yet unknown stressful conditions they encounter after transfusion. We have postulated that the fraction of quickly disappearing erythrocytes is the main cause of the adverse events of transfusions, especially in transfusion-dependent patients. An overload of the reticuloendothelial system could lead to hemolysis and the accumulation of neoantigens, triggering iron acccumulation, inflammation and the formation of alloantibodies.

VOLUME AND DENSITY

Blood bank storage affects volume regulation as indicated, among other things, by the increase in erythrocyte volume already in the first week of storage (Luten et al., 2008b). This is most likely due to the far from physiological composition of most storage solutions, and seems to be readily reversible upon incubation in buffers with a pH and ion concentrations similar to those of the blood. As a complicating factor, storage may affect cation transport and volume regulation differently in young and old erythrocytes (Minetti et al., 2001). There is a striking change in erythrocyte shape with storage, apparently progressing from echinocytes and stomatocytes to an irreversible spherocyte morphology (Blasi et al., 2012). Density separation results in cell fractions in which the increase in density is accompanied by an increase in HbA1c, suggesting that this method is, in principle, also suitable for the study of aging *in vitro* (D'Alessandro et al., 2013b). It is tempting to speculate that the more severely and apparently irreversibly deformed erythrocytes comprise at least a fraction of the removal-prone erythrocytes. Cell volume and density are important factors in deformability. Most data indicate that deformability may decrease with storage time, but to an extent that depends heavily on the methods to measure this parameter. The outcomes of most ektacytometry measurements, for example, are likely to be determined by a decrease in the surface/volume ratio, resulting from vesiculation, and a possibly compensatory decrease in the cellular hemoglobin concentration (Cluitmans et al., 2012).

VESICULATION

The availability of an easily accessible source of relatively pure preparations in the form of erythrocyte concentrates in the blood bank has enabled the generation of many data on storage vesicles (Greenwalt, 2006). However, there is no comprehensive theory on the mechanism of their generation, or on their putative contribution to the adverse side effects of transfusion. Blood bank vesicles are enriched in modified hemoglobin species, as are the erythrocyte-derived vesicles in the plasma (Willekens et al., 2003; Bosman et al., 2008a). Their membrane protein composition, on the other hand, is different from that of erythrocyte-derived vesicles from the plasma, suggesting different vesiculation mechanisms *in vitro* and *in vivo* (Greenwalt, 2006; Bosman et al., 2008b; Kriebardis et al., 2008; Salzer et al., 2008). Also, blood bank vesicles contain more and different plasma-derived proteins, especially immunoglobulins and complement proteins (Bosman et al., 2008b; Kriebardis et al., 2008). The comparison of blood bank vesicles with plasma vesicles is complicated by their accumulation over time in the blood bag, as this results in a heterogeneity in vesicle age that is likely to be much higher than those of freshly isolated vesicles. Also, there is a distinct possibility that the mechanism of vesiculation changes with storage time and storage medium (Greenwalt and Dumaswala, 1988; Bosman et al., 2010a; Sparrow et al., 2013). A more extensive study especially of the biological activity of blood bank vesicles is warranted by their biological activity, and likely effect on the circulation and

immune system of the patient (Gould et al., 2007; Donadee et al., 2011; Kozuma et al., 2011; Xiong et al., 2011).

REMOVAL SIGNALS AND MECHANISMS

Band 3 is very sensitive to proteolytic breakdown, as becomes apparent not only upon immunoblot analysis of purified erythrocytes kept in physiological buffer solutions, but also when stored as whole blood or during storage in blood bank conditions. This sensitivity is most apparent using antibodies against parts of the cytoplasmic domain, suggesting that this domain is especially vulnerable (Bosman et al., 2008a). The changes in this domain are likely to affect the connection between lipid bilayer and the cytoskeleton, and the binding of key enzymes of the glycolysis. Thus, early changes in the cytoplasmic domain may very well be responsible for the storage-associated changes in erythrocyte morphology and metabolism. On the other hand, new metabolomic data support the hypothesis that storage exacerbates the effect of naturally occurring oxidative stress by disturbing the physiological balance between glycolysis, pentose phosphate pathway, and glutathione homeostasis (Gevi et al., 2012; Rinalducci et al., 2012). The apparent reversibility of these changes, especially within the first weeks of storage, may be explained by the redundancy of available binding sites. There are much more band 3 than ankyrin molecules, and the dynamic equilibrium between band 3 monomers, dimers and tetramers may compensate for a small decrease in ankyrin-binding (or enzyme-binding) sites.

The number of IgG molecules that is bound per erythrocyte only slightly increases with storage time. Even after the maximal storage period, the percentage of erythrocytes with an amount of IgG that is sufficient for recognition by macrophages is still very small (Kay, 2005). It is noteworthy that the binding of IgG increases within the first weeks of storage, and then decreases again (Luten et al., 2004; Dinkla et al., 2012a). We speculate that this may be caused by the lysis of the very old erythrocytes early in the storage period, leading to association of spectrin or actin to other, intact cells. This could trigger the binding of low-affinity antibodies, that are normally present in the blood (Garratty, 2005). The number of IgG-containing erythrocytes at the end of the maximal storage time, even after incubation with autologous plasma, is much lower than predicted if the aging process *in vitro* would proceed in an identical manner and with the same speed as it does *in vivo*. If the latter scenario, the percentage of senescent cell antigen-exposing (and thus IgG-containing) erythrocytes would be much higher than observed, even when corrected for the difference in temperature. Therefore, we must conclude that storage in blood bank conditions may have some molecular features of aging *in vivo* (see also **Figure 1**), but that the processes underlying the fast removal of up to 30 percent of the erythrocytes after transfusion cannot be ascribed to a physiological aging process. The recently described, storage-associated increase in the binding of IgG from patients with autoimmune hemolytic anemia to blood bank erythrocytes, shows that aging *in vitro* may, in certain conditions, assume a pathological form (Dinkla et al., 2012a).

Similarly, the very small increase in the number of PS-exposing erythrocytes during storage is far from sufficient to explain the fast removal of up to 30 percent of the stored erythrocytes after transfusion (Verhoeven et al., 2006; Bosman et al., 2011). However, with storage the erythrocytes become very sensitive to stress-induced PS exposure, especially to the near-physiological stress consisting of incubation in a hyperosmotic buffer (Bosman et al., 2011). Together with a concomitant storage-associated increase in susceptibility to generate vesicles (Burger et al., 2013), this observation emphasizes the predisposing, "sublethal" nature of the events that occur in the blood bank.

In a probably analogous manner, there is a detectable change in CD47 conformation during storage only after transfusion *in vitro*, i.e., after incubation of the stored erythrocytes with whole blood (Burger et al., 2012). Also, storage is associated with an increased sensitivity to a band 3 ligand-induced binding of autologous IgG (Bosman et al., 2010b). The storage-associated increase in sensitivity to lipase-induced morphological and biochemical alterations points toward a hitherto little-studied involvement of lipids and lipid organization in erythrocyte vesicle formation and antigen presentation (Salzer et al., 2008; Dinkla et al., 2012b). In general, parameters for a "sublethal injury" such as these are likely to carry more physiologically and clinically relevant weight than osmotic or mechanical fragility (Raval et al., 2010, 2013; Cluitmans et al., 2012).

CONCLUSIONS

The presently available data show that neither our knowledge of the identity of the molecules that signal removal of physiologically aged erythrocytes, nor of the identity of the mechanisms by which they are generated, has progressed much in the last two decennia. One notable exception is the incorporation of vesicle formation in the aging process *in vivo* (Willekens et al., 2008). This is in spite of the strong increase in the number of the phenomena described to accompany especially erythrocyte aging *in vitro*. The recent increase in the number of data on the presence and activity of signaling pathways regulating erythrocyte function, morphology and metabolism, mostly resulting from disease-centered research, opens new possibilities to unravel the mechanisms involved in erythrocyte aging *in vivo* as well as *in vitro*. Regarding the latter, all present data show that storage may be the best available model for studying erythrocyte aging, but do not support the theory that storage in the blood bank is an accelerated form of physiological aging or of a pathological form of aging *in vivo*. This supports the statement that there is still a poor understanding of the storage lesion and its effect on erythrocyte performance. In the view presented here, the most recent data indicate that the processes that occur during storage render the erythrocytes especially vulnerable to the aging phenotype-inducing conditions they encounter after transfusion in the circulation. The increase in susceptibility to stress-induced PS exposure and shrinkage that also occurs during aging *in vivo* (Ghashghaeinia et al., 2012), provides a functional connection between aging *in vitro* and aging *in vivo*. In this context, a recent definition of aging as a "de-tuning of adaptation with increasing age" seems to be directly applicable at the cellular level (Raval et al., 2013).

This leads to the practical conclusion that future research on improving erythrocyte survival after transfusion should concentrate on relating the changes, observed in erythrocytes during

storage, to their resistance to physiological stress that induces the appearance of removal signals after transfusion. This stress could be mechanical, e.g., as experienced during passage through the microcapillaries and the spleen, but also biochemical, e.g., as occurring during the gradual exhaustion of the defense against oxidation and oxidation-induced protein denaturation. Together with the available knowledge on the identity of removal signals, and the identification of the relevant signaling pathways that is likely to happen in the near future, this approach provides a sorely needed, experimental platform to investigate the mechanisms responsible for the dangerous, fast removal of a considerable fraction of the blood bank erythrocytes early after transfusion.

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Mechanisms tagging senescent red blood cells for clearance in healthy humans

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This review focuses on the analysis and evaluation of the diverse senescence markers suggested to prime red blood cells (RBC) for clearance in humans. These tags develop in the course of biochemical and structural alterations accompanying RBC aging, as the decrease of activities of multiple enzymes, the gradual accumulation of oxidative damage, the loss of membrane in form of microvesicles, the redistribution of ions and alterations in cell volume, density, and deformability. The actual tags represent the penultimate galactosyl residues, revealed by desialylation of glycophorins, or the aggregates of the anion exchanger (band 3 protein) to which anti-galactose antibodies bind in the first and anti-band 3 naturally occurring antibodies (NAbs) in the second case. While anti-band 3 NAbs bind to the carbohydrate-free portion of band 3 aggregates in healthy humans, induced anti-lactoferrin antibodies bind to the carbohydrate-containing portion of band 3 and along with anti-band 3 NAbs may accelerated clearance of senescent RBC in patients with anti-neutrophil cytoplasmic antibodies (ANCA). Exoplasmically accessible phosphatidylserine (PS) and the alterations in the interplay between CD47 on RBC and its receptor on macrophages, signal regulatory protein alpha (SIRPalpha protein), were also reported to induce erythrocyte clearance. We discuss the relevance of each mechanism and analyze the strength of the data.

Keywords: human red blood cells, senescence, oxidative stress, hemoglobin, volume, vesicles, naturally occurring antibodies

RED BLOOD CELL AGEING PARAMETERS AND THE CRITERIA OF EVALUATION

Over the years many investigators of red blood cells (RBC) and their biochemical properties have centrifuged whole blood and used the RBC pellet without actively removing leucocytes/platelets, despite their simple and selective depletion has been introduced and recommended almost 40 years ago by Beutler et al. (1976). Many investigators did not add protease inhibitors to the buffers in which RBC and their membranes/extracts were further processed. The omission of these precautions has been and still is the major reason for deviating data. Omission of leucocyte removal has dramatic effects on cell-age sensitive RBC properties, like e.g., hemolysis, echinocytosis, vesicle release, phosphatidylserine (PS) exposure, and band 3 protein clusterization as carefully studied by Antonelou et al. (2012). Thus, leucocyte-depletion renders transfusion of red blood cell units stored for 3–7 days safe (Hod et al., 2011) and ameliorates the effects of long stored blood (Phelan et al., 2010). Hence, the value of a set of data is highly dependent on having taken the first and where possible also the second precaution and we have selected the data for the present review accordingly. Other contradictions originate from a misnomer, for example when authors claim to illustrate a RBC property as a function of cell age, but compare properties of energy-starved RBC with those of freshly isolated ones (Girasole et al., 2012; Kim et al., 2012).

RBC undergo multiple changes while they age *in vivo*. Some of these remain hidden within RBC, others affect the properties of the cell directly, like the loss of cations and the loss of membrane with some hemoglobin by vesiculation that result in an increased cellular density. An increased density implies a higher cell age as has been established by the decreased activities of a number of intracellular enzymes, measured in the lysate (hexokinase, aldolase, pyruvate kinase, glutamate-oxalacetate transaminase) (**Table 1**). The decrease in activity of multiple enzymes is, however, not linear with cell age, but almost exponential from reticulocytes to mature cells [for a comprehensive review of these aspects, see reference Clark (1988)]. This disadvantage has forced many investigators to use as cell age parameters properties that change almost linearly with cell age, as exoplasmically located acetylcholinesterase (Cohen et al., 1976), the RBC creatine content (Fehr and Knob, 1979; Lutz and Fehr, 1979) and the ratio of two Coomassie-blue stainable bands (the 4.1a/4.1b ratio) (Mueller et al., 1987). This ratio illustrates the extent of deamidation of band 4.1b and luckily results in a change of the electrophoretic mobility of the protein in SDS polyacrylamide gels (Inaba and Maede, 1988). The separation of RBC according to their density has been achieved first by centrifuging RBC in an angle rotor where cells circulate to some extent in this highly viscous cell pellet (Murphy, 1973). With the availability of Stractan and Percoll it is the material that establishes a gradient or is arranged in a preformed gradient and allows RBC to better

Table 1 | Changes in RBC properties associated with aging.

RBC property or enzyme studied	Change of activity	Change of activity with cell age phase a/phase b	White cell removal	Species Studied	Data obtained by	References
METABOLIC ENZYMES						
Lactate dehydrogenase/mg protein	–	Constant	Yes	Rabbit	*In vivo* biotinylation	Jindal et al., 1996
Phosphoglycerate kinase/mg protein	–	Constant	Yes	Rabbit	*In vivo* biotinylation	Jindal et al., 1996
Pyruvate kinase /mg protein	–	Constant	Yes	Rabbit	*In vivo* biotinylation	Jindal et al., 1996
Acid phosphatase/mg protein	–	Constant	Yes	Rabbit	*In vivo* biotinylation	Jindal et al., 1996
Pyruvate kinase/mg Hb	D	Exponential	Yes	Human	Density	Haram et al., 1991
Phosphofructokinase (PFK)/Cell number	D	Linear	Yes	Human	Density + Elutriation	Jansen et al., 1986
Glucose-6-phosphate dehydrogenase (G6PD)/cell number	D	Linear	Yes	Human	Density + Elutriation	Jansen et al., 1986
Hexokinase/cell number or mg/Hb	D	Exponential/linear	Yes	Human	Density + Elutriation	Haram et al., 1991; Piomelli and Seaman, 1993
KINASES						
Membrane associated casein kinase/mg protein	D	Linear	Yes	Rabbit	*In vivo* biotinylaiton	Jindal et al., 1996
Membrane casein kinase I/mg protein	D	Linear	Yes	Human	Density	Jindal et al., 1996
Casein kinase I/mg membrane protein	D	Linear	Yes	Rabbit	*In vivo* biotinylaiton	Jindal et al., 1996
Membrane protein kinase C (PKC)/mg protein	I	Increase/linear		Human	Density	Ramachandran and Abraham, 1989
Cytosolic protein kinase C (PKC)/mg protein	D	Exponential	Yes	Rabbit	*In vivo* biotinylation	Jindal et al., 1996
Cytosolic protein kinase C (PKC)/mg protein	D	Exponential	Yes	Human	Density	Jindal et al., 1996
Pyruvate kinase/cell number (PK) or mgHb	D	Exponential	Yes	Human	Density + Elutriation	Jansen et al., 1986; Piomelli and Seaman, 1993
Cytosolic CKII/mg protein	D	Exponential	Yes	Human	*In vivo* biotinylation	Jindal et al., 1996
Cytosolic PKA/mg protein	D	Exponential/constant	Yes	Human/Rabbit	Density	Jindal et al., 1996
AMINO ACID MODIFICATIONS						
Glutamate/oxalacetate transaminase/mg Hb	D	Exponential	Yes	Human	Density	Haram et al., 1991; Piomelli and Seaman, 1993
Aspartate amino transferase (ASAT)/cell number	D	Exponential	Yes	Human	Density + Elutriation	Jansen et al., 1986
AMP deaminase/mg Hb	D	Exponential/constant	Yes	Rabbit	*In vivo* biotinylation	Dale and Norenberg, 1989
MARKERS OF SENESCENCE						
Glutathione reductase (GR)/cell number	D	Linear	Yes	Human	Density + Elutriation	Jansen et al., 1986
HbA1c (glycated Hb) fraction/Whole Hb	I	Linear	–	Human	Biotinylation	Willekens et al., 2003; Cohen et al., 2008
Ratio of content of band 4.1a/4.1b/mg protein	D	Linear	Yes	Many	Density	Mueller et al., 1987; Inaba and Maede, 1988
Acetylcholinesterase units/mg Hb	D	Linear	Yes	Human	Density	Cohen et al., 1976
Creatine/cell number	D	Exponential	Yes	Human	Density	Fehr and Knob, 1979

Changes in activity: I, increase and D, decrease; Phase a, reticulocytes mainly; phase b, mature RBC; Hb, hemoglobin; density, fractionation on the density gradient.

migrate to their actual density (Clark, 1985; Lutz et al., 1992). Aside of density separation aging RBC have been separated from each other by size, using counterflow centrifugation (elutriation), a method that offers a limited yield of separated cells and may best be combined with a preceding density centrifugation (Bosch et al., 1992).

THE CHANGES OCCURRING *IN VIVO*, WHEN LABELED RBC ARE RE-INTRODUCED INTO THE CIRCULATION

By far the most direct method to study *in vivo* aging of RBC is their biotinylation by N-hydroxysuccinimide-biotin and analyzing the properties of the biotinylated RBC during their life span in circulation by collecting the labeled RBC on avidin at various times after injection (Suzuki and Dale, 1987; Christian et al., 1993). The biotin derivative was dissolved in DMSO and a diluted sample was injected intravenously into dogs after bleeding to enhance the portion of young RBC in the labeled population (Christian et al., 1993). In humans bleeding was not an option and biotinylation had to occur *in vitro*. Hence, the fraction of labeled RBC was rather small toward the end of the *in vivo* survival study by 110–126 days (Franco et al., 2013). Nevertheless, it has been possible for the first time to demonstrate that all *in vivo* aged, biotinylated human RBC that were recovered 126 days post injection had increased amounts of membrane-bound IgG, but were not enriched at all in exoplasmically exposed PS (Franco et al., 2013). Similar findings have earlier been reported for dogs having a similar RBC survival time as humans. By 110 days biotinylated RBC carried 7 fold higher amounts of autologous IgG per RBC and massively increased amounts of membrane bound globin (Rettig et al., 1999). Unexpectedly, the density of biotinylated RBC increased primarily during the first 4 weeks of *in vivo* aging, but not or less thereafter as revealed by using preformed density gradients (Franco et al., 2013). Similar results were obtained earlier for biotinylated sickle RBC (Franco et al., 1998). The authors blame the density-separation technique for the unexpected results and suggest that density centrifugation should be combined with elutriation to achieve a better separation according to cell age. However, it cannot be excluded that *ex vivo* biotinylation of RBC in diluted DMSO and several washes had altered the properties of RBC that were not leucocyte-depleted. Nevertheless, analogous results on aging dog RBC confirm the unexpected finding. Dog RBC were *in vivo* biotinylated and revealed during survival signs of an accelerated aging in so far as a classical cell age parameter, the ratio of the band 4.1a/4.1b content had reached its maximum (full deamidation) in the biotinylated RBC portion long before the biotinylated RBC had reached their full survival time (Rettig et al., 1999). It may be possible that the findings were real, implying that a small fraction of aging RBC underwent a terminal density reversal by taking up sodium ions and water, as first described by Bookchin (Bookchin et al., 2000) and discussed in detail by Lew and Tiffert (2013). More studies are needed to clarify whether the terminal density reversal is induced by DMSO or the washes without white cell removal.

In the following chapters we address several parameters delineating age-related changes in healthy human RBC. Among them are oxidative stress, changes in cell volume and density, vesiculation, band 3 clustering, and binding of NAbs.

THE ROLE OF OXIDATIVE STRESS IN RED CELL CLEARANCE

Cell aging is intimately related to the changes in the balance between production of pro-oxidants and their removal by antioxidative enzymes and scavengers to which reduced glutathione (GSH), NADH, NADPH, and ascorbate belong. Gradual accumulation of irreversibly oxidized and denatured proteins, in particular hemoglobin, occurs with ageing (Rifkind and Nagababu, 2013). Changes in activity of multiple enzymes, loss or reorganization of several proteins as well as alterations in plasma membrane lipid composition occur gradually in RBC over 120 days in circulation and are mainly caused by oxidative modifications. *De novo* synthesis of both proteins and lipids is absent in mature RBC. Accordingly, oxidized and denatured proteins accumulate in aging RBC and even more so because aging RBC lose free radical scavengers (Bartosz, 1981).

ANTIOXIDANT CAPACITY OF RBC

Among the antioxidants which prevent oxidation of protein thiols are GSH, NADH, and NADPH. Of these three compounds the half-cell redox potential based on the ratio of GSH to oxidized glutathione (GSSG) is postulated to be a reliable marker of the intracellular redox state (Schafer and Buettner, 2001). Reduced glutathione does not cross the plasma membrane passively and *de novo* synthesis of GSH is the only source of GSH in RBC. Facilitated unidirectional efflux of GSSG from the RBC is mediated by an ATP(GTP)-dependent transporter (RLIP76) and multidrug resistance protein 1 (MRP1) (Srivastava and Beutler, 1969; Bobrowska-Hagerstrand et al., 2001; Sharma et al., 2001). The ability of RBC to synthesize GSH, the presence of enzymes involved in GSH synthesis and GSSG formation (**Figure 1**) have been demonstrated in RBC lysates (Sass, 1968). Two substrates for glutathione synthesis, cysteine and glycine, are transported into the cells whereas glutamate is produced from aspartate and alanine by aspartate aminotransferase and alanine aminotransferase. As follows from the scheme in **Figure 1** *de novo* synthesis of glutathione requires ATP and its reduction from GSSG to GSH requires NADPH.

The intracellular non-protein thiol levels, of which GSH is the major species, decrease substantially during the transformation of reticulocytes to mature RBC, but their concentration remains rather constant thereafter (Magnani et al., 1983; Piccinini et al., 1995). In all these studies the average intracellular GSH and GSSG concentrations were assessed without discriminating between the ratio of GSH to GSSG in the pre-membrane pool and that in the cytosolic core. Since the oxidative stress in aging RBC is particularly severe at the membrane, these measurements may not precisely reflect the aging-related shift in the redox state. The activity of γ-glutamylcysteine synthetase and that of GSH synthetase, two major enzymes involved in glutathione synthesis (**Figure 1**), remain unchanged in RBC with increasing density (Minnich et al., 1971) and get suppressed only in the cells showing the highest density on the Stractan gradient (Piccinini et al., 1995). The activities of key glycolytic enzymes hexokinase, pyruvate kinase, and glucose-6-phosphate dehydrogenase,

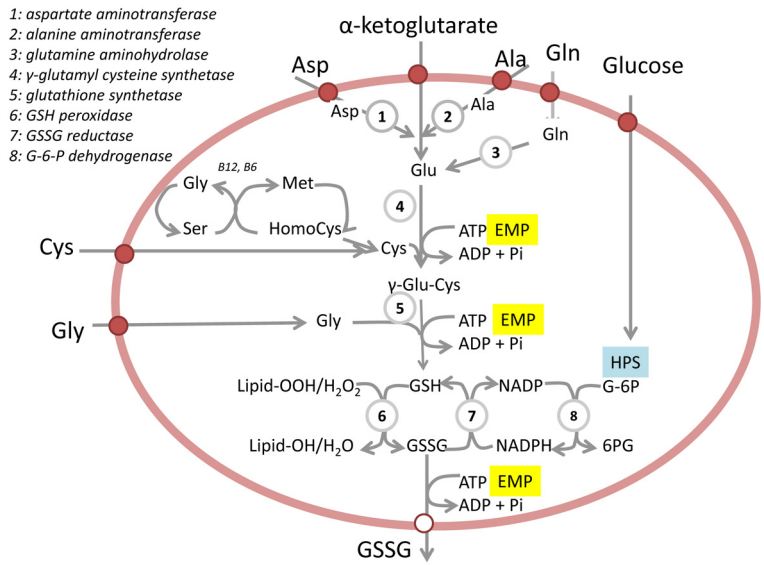

FIGURE 1 | Glutathione production and turnover in RBC. Schematically presented are substrate delivery, glutathione synthesis, and glutathione handling in RBC. EMP stands for Embden-Meyerhof pathway (anaerobic glycolysis). HPS denotes hexose monophosphate shunt (pentose phosphate pathway). G-6P and 6 PG stand for glucose-6-phosphate and 6-phosphogluconolacton respectively. Glu, Gln, Gly, Cys, Ala, and Asp stand for glutamate, glutamine, glycine, cysteine, alanine, and aspartate respectively.

as well as that of aspartate aminotransferase which provides glutamate for GSH synthesis, were the highest in the low density fraction and progressively decreased with an increase in red cell density [(Fornaini et al., 1985; Jansen et al., 1986) and **Table 1**].

Glucose transport across the RBC membrane decreases with cell age (Bosman and Kay, 1990). Young (least dense) RBC metabolize 2.5 times more glucose than old ones. On the other hand, the amount of glucose utilized via the hexose monophosphate shunt does not show any age dependence. Intracellular ATP levels drop by 30–40% following the down-regulation of Embden-Meyerhof (EMP) pathway (Cohen et al., 1976; Magnani et al., 1983), contributing to a shortage of GSH production in the densest RBC fraction.

RBC possess an efficient enzymatic machinery to process and detoxify reactive oxygen and nitrogen species, including superoxide dismutase (SOD1), catalase, peroxidases as well as glutathione peroxidase, peroxiredoxin 2 and glutaredoxin 1, which reverse oxidative thiol modifications on proteins and preserve enzyme activities from oxidative inactivation. The activities of some of these anti-oxidants, such as SOD1 and catalase decline during aging (Bartosz et al., 1978; Bartosz, 1980, 1981; Bartkowiak et al., 1983). Even extracellular SOD isozymes, including mitochondrial SOD2 (presumably from the endothelial cells), participate in detoxifying free radicals that cause RBC oxidation, as SOD2-deficient animals presented with higher rates of hemoglobin oxidation than controls (Mohanty et al., 2013). Further studies using knockout animals indicate that at least in rodents peroxiredoxin 2 and glutaredoxin 1 are the major enzymes detoxifying endogenous H_2O_2 in RBC (Lee et al., 2003; Johnson et al., 2010). Catalase on the contrary takes over detoxification of H_2O_2 produced externally (Johnson et al., 2010). RBC age-dependent changes in peroxiredoxin 2 and glutaredoxin 1 activities have not been studied yet.

SOURCES OF OXIDATIVE EQUIVALENTS IN SENESCENT RBC

Cell age-related oxidation is largely a membrane-localized event, because free radical generators are compartmentalized. Most of them are localized within the membrane or are attached to membrane proteins from the cytosolic side, whereas enzymes detoxifying them as well as low molecular weight thiols are randomly distributed within the cytosol.

Auto-oxidation of hemoglobin is considered to be the major source of superoxide anion production in senescent RBC. Reduction of dioxygen to O_2^- is associated with the generation of methemoglobin. Ferric iron of methemoglobin is then reduced to the ferrous state by hemoglobin reductase which uses NADH as a substrate. The methemoglobin concentration was shown to increase linearly with increasing RBC density (Imanishi et al., 1985; Rettig et al., 1999). *In vitro* studies of the hemoglobin auto-oxidation revealed that the reaction is slow ($k = 0.0115\,h^{-1}$) under normoxic conditions (Nagababu et al., 2002), but is facilitated dramatically upon partial deoxygenation (Abugo and Rifkind, 1994) and is maximal at pO_2 of 1.33 kPa (Rifkind et al., 2004). Deoxyhemoglobin readily binds to the cytosolic domain of band 3 (Walder et al., 1984). This suggests that the levels of deoxyhemoglobin at the membrane surface exceed those in the cytosol. Furthermore, aging of RBC is associated with an increase in spectrin-hemoglobin complexes (Snyder et al., 1983) which contribute to an increased rigidigty of senescent RBC in dense fractions (Fortier et al., 1988). Accumulation of hemoglobin at the membrane surface and its auto-oxidation results in production of superoxide anion. Two further classes of enzymes which contribute to superoxide production are NADPH

oxidases of which several isoforms are present in RBC (George et al., 2013) and endothelial NO synthase which is present in human and mouse RBC (Kleinbongard et al., 2006) and produces O_2^- when L-arginine levels are low (Mihov et al., 2009).

Superoxide anion has a half-life of 10^{-6} s and undergoes a number of transformations depending on the availability of NO, superoxide dismutase (SOD), and H_2O_2. Interaction of O_2^- with NO ($k = 4–610^9\,M^{-1}s^{-1}$) is four orders of magnitude faster than dismutation of O_2^- to H_2O_2 catalyzed by SOD ($k = 210^5\,M^{-1}s^{-1}$). Thus, formation of peroxynitrite ($ONOO^-$) from O_2^- dominates over the transformation of O_2^- to H_2O_2 catalyzed by superoxide dismutase if NO is available (Borges-Alvarez et al., 2012). Peroxynitrite generated in this manner is believed to be a potent mediator of oxidative stress in RBC (Minetti et al., 2008; Rifkind and Nagababu, 2013). However, aging of RBC is not associated with an accumulation of nitrated tyrosine, the product of peroxynitrite reacting with membrane proteins or hemoglobin (Kikugawa et al., 2000). It is suggested that peroxynitrite production in circulating RBC is minimal due to the low abundance of deoxyhemoglobin (Winslow and Intaglietta, 2008). Thus, the importance of peroxynitrite as a mediator of oxidative stress in aging RBC remains questionable. NO may be viewed as a scavenger of superoxide radicals and therefore as a member of the antioxidative defense system. This defense system is challenged particularly under hypoxic conditions when partially oxygenated hemoglobin prone to auto-oxidation is formed. Deoxygenated hemoglobin has been shown to function as nitrite reductase transforming nitrite to NO being itself oxidized to methemoglobin (Gladwin and Kim-Shapiro, 2008).

Endogenous hydrogen peroxide formed by a SOD1-catalysed reaction as well as exogenous H_2O_2, diffusing into RBC from plasma, may be detoxified by catalase or peroxidase. However, when ferrous or ferric ions are available they catalyze reactions known as Haber-Weiss cycle in which hydroxyl radicals (HO) are formed. The hydroxyl radical has a half-life of 10^{-9} s and is extremely reactive and pro-oxidative. Iron ions are becoming accessible in the pre-membrane space during the process of oxidation and denaturation of membrane-bound hemoglobin occurring with aging of RBC (Low et al., 1985). Hemichrome accumulation and binding to the cytosolic domain of band 3 protein is a hallmark of RBC senescence.

TARGETS OF OXIDANTS

RBC are one of the models of choice to monitor the effects of oxidants on proteins and lipids [e.g., Di Simplicio et al., 1998; Minetti et al., 2008]. However, relatively few studies refer to the monitoring of thiol modifications during ageing of RBC *in vivo* in healthy humans.

Hemoglobin, being the most abundant (98% of total protein content) protein in RBC, is the main generator of reactive oxygen species, the main target of oxidative damage and also a scavenger of free radicals. Detoxification of free radicals by hemoglobin is associated with the production of met-hemoglobin and oxidation of a single cysteine residue present in position 93 of the beta chain (Vitturi et al., 2013). The generated methemoglobin releases iron as Fe^{+3} in a chelatable form that can further propagate oxidative damage and induce binding of autologous IgG. Ferrali et al. synthesized an aromatic iron-chelator and applied it to mice in which oxidative damage and iron release were induced by a phenylhydrazine treatment. The RBC from animals treated with the chelator were protected from oxidative damage and from binding of autologous IgG (Ferrali et al., 2000).

Band 3 protein is also a target of oxidation, particularly when it has formed complexes with oxidized and denatured hemoglobin, hemichrome (Mannu et al., 1995). Accumulation of membrane-associated oxidized hemoglobin is considered to be one of the major (but not the only) trigger of band 3 cluster formation (Low et al., 1985; Schlüter and Drenckhahn, 1986). In addition, oxidation is a trigger for activation of src tyrosine kinases (Mallozzi et al., 2001) and inhibition of tyrosine phosphatases (Zipser et al., 2002), resulting in a cumulative tyrosine hyperphosphorylation of membrane target proteins. The src kinases including syk and lyn kinases phosphorylate tyrosines 8 and 21 of the cytosolic domain of the band 3 protein, thereby facilitating formation of high-molecular-mass band 3 aggregates (Pantaleo et al., 2009). Oxidation and poor glycosylation of band 3 further facilitates clustering of this protein (Pantaleo et al., 2009). Its tyrosine phosphorylation markedly reduces the affinity of band 3 to ankyrin, causing destabilization of the band 3-cytoskeleton interaction, increases the lateral mobility of band 3 within the membrane and induces vesiculation (Ferru et al., 2011). Both srk kinases syk and lyn and the tyrosine phosphatase PTP1B are redox-sensitive, be it because they possess cysteine residues in the kinase domains or indirectly, because their activity is controlled by the redox- and calcium-sensitive phosphorylation steps mediated in particular by protein kinase C alpha (Bordin et al., 2005; Knock and Ward, 2011).

CHANGES IN ACTIVITY OF ION TRANSPORTER DURING RBC SENESCENCE

Transformation from reticulocytes to mature RBC and ageing of the latter is associated with radical re-organizations of the plasma membrane. Reduction in membrane surface by means of exocytosis during maturation of reticulocytes enables the cells to reduce or completely eliminate a number of receptors and ion transporters which play an active role in differentiation of erythroid precursor cells and are no longer required by mature RBC. Among the most well-known is the transferrin receptor. The number of copies per cell of Na,K-ATPase, Na/glycine transporter (Blostein and Grafova, 1987), nucleoside transporter, and K-Cl cotransporter (Canessa et al., 1987; Ellory et al., 1991) is reduced and renders the cell less energy-demanding and more stable.

Several ion transporters that are not extruded in exosomes undergo an age-dependent inactivation. The activity of the Gardos channel is reduced with ageing (Tiffert et al., 2007). Similarly, the number of copies of N-methyl D-aspartate (NMDA) receptors, non-selective cation channels mediating Ca^{2+} uptake into RBC, also decreases with cell age (Makhro et al., 2013). Plasma membrane Ca^{2+} pump was also reported to decline with cell age (Lucas et al., 1988; Samaja et al., 1989).

These conclusions may not necessarily apply to all cells within the light, medium or dense fractions of RBC, but to a majority of them. Recently extreme heterogeneity in responses of RBC to glutamate and homocysteic acid (Makhro et al., 2010, 2013),

prostaglandin E2 (Kaestner et al., 2004), LPA (Wagner-Britz et al., 2013) has been recognized. This heterogeneity results from the inter-cellular variability in abundance of the corresponding ion transport pathways [NMDA receptors, voltage-gated Ca^{2+} channels (Cav2.1), and LPA receptors] in RBC of healthy humans (Makhro et al., 2013; Wagner-Britz et al., 2013).

In the following section the impact of vesicle release on RBC aging will be discussed. This process of membrane loss also contributes to the increase in density of senescent cells.

RELEASE OF MICROVESICLES AND NANOVESICLES FROM AGING RBC

It is known for many years that senescent RBC are smaller, denser than young cells and have lost membrane and hemoglobin. Nevertheless, many investigators have quantified N-acetylneuraminic acid (sialic acid) per young and per old RBC and then claimed that sialic acid decreased with cell age and the exposed remainder may act as a senescent cell marker, capable to trigger/mediate their selective removal (Gutowski et al., 1991; Bratosin et al., 1995). When the content of sialic acid was referred to a measure of the number of integral membrane proteins on young and old RBC, it was with ± 1.5% the same (Lutz and Fehr, 1979) and the cellular electrophoretic mobility remained unchanged during RBC aging (Luner et al., 1977). Despite the electrophoretic mobility remains unchanged, aging RBC loose a substantial portion of their membrane and content in form of microvesicles [reviewed in references Greenwalt (2006), Tissot et al. (2010)]. For a long time microvesicles were not in the focus of research, because those that are released *in vivo* are rapidly cleared and their relation to those from stored RBC was unclear. This situation has drastically changed since it has become obvious that the microvesicles from stored RBC have deleterious effects in transfused patients [for a review see Tissot et al. (2010)]. One of the deleterious effects comes from priming the respiratory burst of neutrophils (Jank and Salzer, 2011), which was, however, far more pronounced by microvesicles from RBC that were not leucocyte-depleted (Cardo et al., 2008).

The shedding of microvesicles from *in vivo* aging RBC was first illustrated by Dumaswala and Greenwalt (1984). By that time two types of *in vitro* RBC vesiculations had already been discovered as laboratory phenomena: Allan and coworkers had characterized vesicles released from RBC incubated with Ca^{2+} and ionophore (Allan and Michell, 1977) and Lutz and coworkers had studied vesicles released from ATP-depleted RBC (Lutz et al., 1977). Both types of vesicles contain hemoglobin and their membranes are enriched about 2-fold in exoplasmic acetylcholinesterase, lack spectrin and ankyin, but retain the integral membrane proteins band 3 and glycophorin to about 50%. Generation of microvesicles from stored RBC was also described in the 70s for storage in ACD (acid-citrate-dextrose) by Rumsby et al. (1977). These authors noted that stored RBC release hemoglobin-filled microvesicles that are depleted of spectrin, but contain integral membrane proteins. More recent data on the microvesicle release from stored and from Ca^{2+}-loaded RBC that were leucocyte-depleted, have yielded the following insights. At the onset of RBC storage the released microvesicles differ in composition from those released from Ca^{2+}-loaded RBC. After prolonged storage (beyond 21 days), when the ATP-content of stored RBC decreases rapidly, the total amount of released vesicular proteins increases exponentially, reaching 10 times the amount by 50 days as compared to 14 days of storage and their composition becomes comparable to that of Ca^{2+}-induced vesicles (Salzer et al., 2008). It is possible that this late change in amount and properties of microvesicles from stored RBC may be the major reason for why long stored blood units were primarily responsible for the deleterious effects in transfused patients.

RBC shrinkage and echinocytosis precede the release of microvesicles. It is known that budding and the release of microvesicles from RBC incubated with Ca^{2+} and ionophore are induced by the formation of diacylglycerol (Allan and Michell, 1977). Microvesicle generation from ATP-depleted RBC also correlates with the breakdown of polyphosphoinositides to diacylglycerol on the inner monolayer (Müller et al., 1981). The diffusible diacylglycerol partitions into the outer monolayer and thereby contributes to membrane budding. Nevertheless, membrane budding and shedding of microvesicles yet require other changes, because 10 mM EDTA inhibited microvesicle release by 75% without affecting diacylglycerol production (Müller et al., 1981). Indeed, membrane budding is further dependent on exoplasmic exposure of phosphatidylserine (PS), a negatively charged phospholipid that normally is exclusively localized on the inner monolayer. Its exposure on the outer monolayer depends on activation of the scramblase by intracellular Ca^{2+}, which is induced by ionophore (Nguyen et al., 2011) or by lysophosphatidic acid during RBC storage, but less upon prestorage leukodepletion (Nagura et al., 2013). It was further noted that prolonged storage of leucocyte-depleted RBC increases the susceptibility of RBC to stress-induced loss of phospholipid asymmetry. The effect was most pronounced for old RBC when experimentally evoked by a hyperosmotic shock, such that PS exposure increased 10-fold by 4 weeks storage and more so in old RBC (Bosman et al., 2011). Strong arguments in favor of PS exposure being required for microvesicle release come from (1) a bleeding disorder, the Scott syndrome, where RBC neither translocate PS across the membrane nor release microvesicles (Bevers et al., 1992) and (2) the finding that a specific inhibitor of the scramblase (R5421) inhibits both PS exposure and microvesicle release to more than 50% (Gonzalez et al., 2009).

The regions on RBC that become enriched in diacylglycerol and PS are associated with lipid rafts. Lipid rafts are microdomains in the plasmamembrane, rich in cholesterol and glycosphingolipids and contain unique proteins that differ in the two types of rafts known to exist in RBC. One type contains primarily stomatin and the flotillins (flotillin1 and 2). While the palmitoylated, hydrophobic stomatin is an integral membrane protein, the flotillins are peripheral membrane proteins with hydrophobic domains capable of associating with the bilayer (Salzer et al., 2002, 2008). Microvesicles (160 nm diameter) from RBC stored for more than 20 days (Kriebardis et al., 2008) and from Ca^{2+} loaded RBC (Salzer et al., 2002) contain band 3, are enriched in acetylcholinesterase and in stomatin (2-fold as compared to the original membrane), but threefold depleted of flotillin 2 that remains in the residual RBC membrane (Salzer et al., 2008). The thrombogenic activity of microvesicles released

from stored RBC or from Ca^{2+} and ionophore treated RBC is comparable (Salzer et al., 2008). Microvesicles from long stored RBC contain heavily aggregated hemoglobin, band 3 aggregates and increasing amounts of autologous IgG (Kriebardis et al., 2008), while the residual RBC reveal a decrease in band 3 content, but yet a substantial increase in bound IgG (Kriebardis et al., 2007). Both types of microvesicles also contain small amounts of synexin and sorcin. The two peripheral membrane proteins, synexin and sorcin are predominantly associated with the second type of lipid raft in RBC and become the major proteins in the Ca^{2+}-induced nanovesicles (60 nm diameter) (Salzer et al., 2002). Nanovesicles lack band 3, contain little stomatin, but are highly enriched in the two peripheral proteins synexin and sorcin, two proteins that were not known to exist in RBC (Salzer et al., 2002). Synexin is an annexin-like protein and binds in a Ca^{2+} dependent manner to sorcin and the cytoplasmic side of the budding membrane of nanovesicles. In other cells the two proteins play important roles in fusion of lamellar bodies (Sen et al., 1997).

The sequence of events in microvesicle and nanovesicle formation and release illustrates that cellular aging as evident from increasing amounts of oxidized hemoglobin, aggregated band 3 protein and surface-bound IgG appears to induce an entrapment of these irreversible endproducts in lipid raft-containing microvesicles that are rapidly cleared. The rapid clearance of these microvesicles occurs most likely by Kupfer cells and other macrophages via recognition of exposed PS, as established in an animal model using rats (Willekens et al., 2005). The selective release of vesicles containing aggregated band 3 with bound naturally occurring antibodies evidently prevents a premature recognition of the aging RBC by phagocytes.

NATURALLY OCCURRING ANTI-BAND 3 ANTIBODIES AND COMPLEMENT IN CLEARANCE OF SENESCENT HUMAN RBC

When Kay published her first paper on the "mechanism of removal of senescent cells by human macrophages" in 1975 it was the first contribution that addressed the selective clearance of *in vivo* aged human RBC (Kay, 1975). Senescent but not young RBC, as obtained by density separation, are phagocytosed by macrophages, while phagocytosis of *in vitro* aged RBC requires opsonization with autologous IgG. IgG eluted from senescent RBC induces phagocytosis of *in vitro* stored young RBC (Kay, 1978). These findings demonstrated that "opsonization of aging RBC by autologous IgG" represents the effect of "beneficial autoantibodies," of "physiologic autoantibodies" that were also called natural antibodies or naturally occurring (auto)antibodies (NAbs). NAbs with beneficial roles had already been observed in 1942 (Kidd and Friedewald, 1942), but went almost unnoticed during the rapid development of classical immunology in the 60 and 70 s. This situation changed in the early 80 s with the description of NAbs against nine common human antigens (Guilbert et al., 1982) and the availability of the immunoblotting technique to visualize binding of whole IgG to individual membrane proteins (Towbin et al., 1979) and to embark on the affinity purification of NAbs to RBC membrane proteins like spectrin (Lutz and Wipf, 1982) and band 3 (Lutz et al., 1984).

In trying to identify the antigen exposed on aged RBC Kay carried out affinity purification of RBC proteins on immobilized IgG from senescent RBC and reported that this IgG NAb binds to a 62 kDa protein of old RBC (Kay, 1981). At the same time Lutz had evidence for band 3 oligomers to represent the antigen to which autologous IgG binds to (Lutz, 1981). The immune precipitate obtained with second antibody from detergent extracts of ^{125}I-iodinated RBC contained material at 100 and 200 kDa and shared iodinated peptide maps with that of band 3 protein. Analogous precipitates from chymotrypsin-treated RBC yielded primarily a labeled 65 kDa fragment, implying that the intact antigen was band 3 (the two chymotryptic fragments of band 3 were ascribed the MW of 65 and 38 kDa to the carbohydrate-containing fragment, later with sequence data available these fragments were named 55 and 35 kDa fragment). Lutz and coworkers could establish that the antigenic site preexists in band 3 protein, but becomes accessible for NAb binding upon band 3 clusterization e.g., on spectrin-free vesicles in which band 3 is laterally mobile. This overview was concluded by suggesting that also in Kay's experiments the RBC-specific NAb may have bound to band 3/band 3 aggregates, but ended up in a 62 kDa fragment because of proteolysis (Lutz, 1981). The above mentioned data could not be published in a prestigious journal, because the existence of anti-band 3 NAbs had not yet been proven, though immunoblots with whole IgG revealed not only binding to both spectrin band 1 and 2 but also to band 3 (Lutz and Wipf, 1982). Eventually the entire information was published in pieces: Binding of autologous IgG was 14 times higher to spectrin-free vesicles than to ATP-maintaining RBC (Müller and Lutz, 1983), implying that NAb binding increases with oligomerization of integral membrane proteins. A detailed study of the glycoprotein topology on human RBC after an extensive aminogroup supplementation further showed that the 10^6 band 3 molecules exist either as monomers or non-crosslinkable dimers of which the crosslinkable portion is minute, but increases at a confidence level of 0.06 by 0.4% from young to senescent cells (Schweizer et al., 1982). By leucocyte-depletion of RBC, by inhibiting proteases with phenylmethyl sulfonyl chloride (PMSF), by surface-^{125}I - iodinating young and old RBC, by extraction with Triton and addition of a second antibody it was demonstrated that preexisting immune complexes contained band 3 monomers and oligomers in samples from old but not young RBC (Lutz and Stringaro-Wipf, 1983). Furthermore, preincubation of extracts from young RBC with autologous IgG resulted in immunoprecipitation of band 3, again suggesting the preexistence of antigenic sites in young RBC. Finally, anti-band 3 NAbs were purified from IgG of individuals and pooled IgG (Sandoglobulin) and characterized (Lutz et al., 1984). The antigenic site of anti-band 3 NAbs is located within the 65 kDa, but not the 38 kDa chymotryptic fragment of band 3 and antigenic band 3 protein is equally present on young and old RBC, implying that exposure requires an altered accessibility rather than an enzymatic generation of antigenic sites as suggested by Kay (Kay and Goodman, 1984). Indeed binding of purified anti-band 3 NAbs was about 10 times higher to band 3 oligomers than to monomers (Lutz et al., 1984).

In 1983 Kay followed the initial observation of Lutz et al. and reported that the senescent cell antigen is immunologically related to band 3, because not only the 62 kDa protein, but also intact band 3 inhibited phagocytosis of RBC incubated with IgG

eluted from senescent RBC (Kay et al., 1983). In contrast to earlier work from her group, RBC studied in this paper were leucocyte-depleted and proteases inhibited in extracts. Correspondingly the stained band pattern of RBC ghosts did not reveal the 62 kDa fragment [for comparison see reference Kay (1981)]. The anti-senescent cell IgG was said to bind to both chymotryptic fragments from band 3, though the blot showed almost exclusive binding to the larger chymotryptic fragment (Kay, 1984). While this disagreement on anti-band 3 specificity continued (Kay et al., 1988; Kay, 1992), others built on the findings of Lutz et al. and extended their own studies on cell age dependent hemichrome binding to band 3 protein (Waugh and Low, 1985) by asking whether the clustering of band 3, which is induced by denatured hemoglobin might also promote binding of autologous IgG (Low et al., 1985). Indeed, oxidative damage induced by a phenylhydrazine treatment of RBC, known to result in hemichromes, greatly stimulated binding of autologous IgG and IgG binding colocalized with band 3 protein (Low et al., 1985). Sorette and Clark raised their doubts on the treatment with phenylhydrazine to simulate cell aging and showed that the highest IgG binding was to RBC with membrane lesions (Sorette and Clark, 1991). Drenckhahn a former collaborator of Low continued their joint studies and demonstrated that RBC of patients with unstable, oxidation-sensitive forms of hemoglobin (Heinz body anemia, hemoglobin Köln, and sickle cell anemia) revealed a co-clustering of denatured hemoglobin, of band 3 protein and of RBC-bound immunoglobulins, without having to pretreat RBC with an exogenous oxidizing agent (Schlüter and Drenckhahn, 1986). Low's group then isolated the densest human RBC and showed it contained 6 times more membrane bound hemichromes and 10 times more surface-bound autologous IgG than other RBC fractions of lower density (Kannan et al., 1991). They even managed to enrich the microscopic aggregates comprised of hemichromes, band 3 and spectrin, constituting 0.09% of the membrane protein, but carrying 55% of the total cell-bound IgG.

On a meeting in Israel Arese and Lutz got to know each other and decided to collaborate by using the phagocytosis assay with diamide-treated human RBC developed in Arese's lab (Bussolino et al., 1987) to investigate the functional properties of purified anti-band 3 NAbs. Anti-band 3 NAbs purified from pooled human IgG (Sandoglobulin) bound to SS group-containing band 3 oligomers and stimulated phagocytosis of diamide-treated RBC maximally at 10–20 μg/ml (Lutz et al., 1987). Largely unexpected was that efficient phagocytosis required C3b deposition, unless anti-band 3 NAbs were added at 20–100 times the physiological concentration. C3b binding to diamide-treated RBC was about two orders of magnitude higher than that of anti-band 3 NAbs and even occurred under alternative complement pathway conditions (Lutz et al., 1987). Likewise, senescent RBC having a five-fold lower creatine content than young RBC not only contained significantly more IgG, but also SDS-resistant complexes comprised of IgG and C3b as verified by immunoblotting with anti-IgG and anti-C3c (Lutz et al., 1988). Then, by quantifying the binding of labeled anti-band 3 NAbs to C3 it became obvious that anti-band 3 NAbs have a unique affinity for C3, while anti-spectrin NAbs not (Lutz et al., 1989). The weak affinity for C3 ($2–3 \times 10^5$ l/mol) at a site independent of the antigen binding domain is about 100 times higher than that of whole IgG, known to have a weak affinity for C3 (Lutz et al., 1993b). This affinity for C3 is responsible for the preferential formation of C3b-IgG and $C3b_2$-IgG complexes during complement activation (Lutz et al., 1993c). Years later Jelezarova et al. verified that all such complexes contain dimeric C3b ($C3b_2$-IgG), ester-bonded to one heavy chain of IgG (Jelezarova et al., 2003). It is the dimeric C3b within these complexes that renders them efficient activators of the alternative complement pathway. Thus, the ability of certain low affinity NAbs, like anti-band 3, to form such complexes during complement activation renders them far more efficient opsonins than judged from the number of bound antibody. Frank and collaborators had earlier found that certain induced IgG antibodies to bacteria also formed covalent complexes with C3b (C3b-IgG), which rendered these IgG molecules 3–4 fold more effective in stimulating complement deposition (Joiner et al., 1985).

To clarify the homeostatic role of purified anti-band 3 NAbs the survival of untreated and diamide-treated RBC was investigated in guinea pigs. In normal, but not in C3-deficient guinea pigs human anti-band 3 NAb binding significantly accelerated the clearance of diamide-treated guinea pig RBC (Giger et al., 1995). Likewise, a pretreatment of the animals with 200 μg of human band 3 protein slowed down the clearance of diamide-treated guinea pig RBC to the extent observed without anti-band 3 NAbs. In support of the role of complement Turrini et al. used either $ZnCl_2$, acridine orange or melittin to cluster integral membrane proteins and determined binding of autologous IgG, complement C3c and quantified phagocytosis. The authors could confirm and extend the observations from the group of Lutz in so far as the clustering agent was only effective upon subsequent crosslinking of aggregated band 3 protein and only then induced binding of autologous IgG, deposition of C3c and phagocytosis (Turrini et al., 1991). IgG eluted following disaggregation of band 3 oligomers bound almost exclusively to band 3, its dimer and oligomers (Turrini et al., 1993). Disulfide-cross-linked band 3 dimers are indeed the minimal band 3 aggregates with enhanced affinity for anti-band 3 NAbs (Turrini et al., 1994). Similar conclusions were drawn by Beppu et al., using three different approaches to oxidize human RBC. All pretreatments increased binding of autologous IgG as well as of purified anti-band 3 NAbs and binding was inhibited by purified band 3 protein or by restoration of the protein SH groups (Beppu et al., 1990).

Oxidation or a treatment with a clustering agent and cross-linking of the generated clusters evidently simulates the prerequisites for clearance of RBC, but does not explain how band 3 clusters are formed during *in vivo* aging. Several authors have focused on the potential role of superoxide and NO, known to form peroxynitrite that induces lipid peroxidation and oxidizes hemoglobin to methemoglobin (Matarrese et al., 2005). Oxidative damage by peroxynitrite induces tyrosine phosphorylation of band 3 protein by several orders of magnitude and this phosphorylation enables the dissociation of band 3 from the spectrin-actin skeleton by lowering its affinity for ankyrin, whereby its cross-linkability and in plane diffusion are elevated (Ferru et al., 2011). Despite peroxynitrite can induce the

oxidation of hemoglobin, can provide the prerequisite for band 3 protein oligomerization and can enable exoplasmic PS exposure, all these alterations are reversed by reactivation of glycolysis (Pietraforte et al., 2007). This implies that damage is minimal in aging RBC as long as ATP levels are maintained. Hence, peroxynitrite may contribute to oxidative damage in microvesicles, but not in aging RBC that maintain their ATP concentration at a high level and do not expose PS exoplasmically. An elegant recent report has finally brought the answer. Arashiki et al. have shown that efficient binding of methemoglobin to band 3 protein, requires a preceding peroxidation of the cytoplasmic portion of band 3 protein (Arashiki et al., 2013). Peroxidation of the cytoplasmic portion of band 3 protein results in carbonylation of this domain and this in turn enhances methemoglobin binding 5–7 fold. Bound methemoglobin then induces a conformational change which displaces ankyrin and gives rise to band 3 cluster formation.

ANTI-BAND 3 NAbs AND INDUCED ANTI-LACTOFERRIN ANTIBODIES

Anti-band 3 NAbs, as isolated from plasma of healthy blood donors with blood group O or from Sandoglobulin bound to band 3 and exclusively to the 68/55 kDa chymotryptic fragment of band 3, but not to the carbohydrate-containing, 38/35 kDa fragment (Lutz et al., 1984). Eight years later Beppu reported that their anti-band 3 NAbs bind to the sialylated N-acetyllactosaminyl carbohydrate group localized within the 38 kDa fragment of band 3 (Beppu et al., 1992; Ando et al., 1994). Lutz et al. reinvestigated the specificity of anti-band 3 NAbs purified from pooled human IgG as found in Sandoglobulin. Anti-band 3 NAbs as prepared originally bound to band 3 and weakly to the cytoskeletal proteins band 4.2, 5 and spectrin. In the reinvestigation anti-band 3 NAbs were further purified by absorption on heat aggregated human IgG to deplete of anti-idiotypes. Anti-band 3 NAbs obtained in this manner bound on blots exclusively to band 3 and to the 55 kDa chymotryptic fragment of band 3, but not the 38 kDa chymotryptic fragment of band 3 (Lutz et al., 1993a). A detailed analysis further showed that binding of anti-band 3 NAbs to blotted band 3 protein from RBC membranes was neither inhibited by pretreating RBC with neuraminidase nor endo-β-galactosidase. In addition, its binding to band 3 and to the 55 kDa fragment of band 3 was not inhibited at all by 10 μg/ml lactoferrin (Lutz et al., 1993a). This was in complete contrast to the properties of Beppu's "anti-band 3 antibodies," for which these authors showed an 80% inhibition of binding to oxidatively stressed RBC by 10 μg/ml lactoferrin (Beppu et al., 1992). The consequence was that many investigators in the RBC field considered the specificity of anti-band 3 NAbs as unresolved, as remaining controversial.

The second round of studying anti-band 3 specificity confirmed the initial characterization of anti-band 3 NAb preparations of Lutz and suggested that Beppu's group must have used a different type of starting material that upon purification on immobilized band 3 protein yielded anti-N-acetyllactosaminyl-specific IgG and anti-band 3 NAbs. This possibility was later rendered even more likely, when Ando et al. (1996) showed that 70% of their anti-band 3 antibodies bound to lactoferrin and 30% to a non-glycosylated portion of band 3 protein. The source of their starting material was ill defined: was serum from persons with blood group AB, (Beppu et al., 1992), blood group B (Beppu et al., 1990), normal adults (Fujino et al., 2000) and was further treated for 30 min at 56°C, at least in the first paper (Beppu et al., 1990). Nevertheless, none of these starting materials could explain the copurification of the two types of antibodies on band 3 protein. Years later, with additional clinical information available, Lutz came up with the suggestion that Beppu's group must have used serum/plasma from patients with one of the many autoimmune diseases that are characterized by anti-neutrophil cytoplasmic antibodies (ANCA) (Lutz, 2012). IgG anti-lactoferrin is one of the induced autoantibodies in patients with rheumatoid arthritis (Kida et al., 2011), systemic lupus erythematosus (Caccavo et al., 2005), ulcerative colitis (Peen et al., 1993; Teegen et al., 2009), cholangitis (Muratori et al., 2001), and many other chronic diseases. In contrast to this, healthy humans have no IgG anti-lactoferrin at all, as verified by ELISA on 34 (Caccavo et al., 2005) and 36 (Chikazawa et al., 2000) serum samples and by immunoblotting (Nässberger et al., 1994). Thus, the purified IgG antibody preparation from Beppu's group should not have been named "anti-band 3 Nab." Their preparation is evidently a mixture of NAbs with an induced autoantibody.

On the other hand, the functional properties that Beppu's group has described for this mixture of anti-band 3 and anti-lactoferrin reveal an unexpected implication, namely that not only anti-band 3 NAbs, but also anti-lactoferrin antibodies bind to oxidatively stressed and aging RBC and together increase opsonization with IgG and complement (Beppu et al., 1996; Ando et al., 1997). The consequence is that patients with induced IgG anti-lactoferrin antibodies may suffer from anemia as was found for example in 6 SLE patients having no other ANCA type antibody, but anti-lactoferrin (Manolova, 2003). In other studies anemia accompanying the presence of anti-lactoferrin antibodies was characterized on the basis of diminished hemoglobin concentrations, elevated erythrocyte sedimentation rates (ESR) and/or a higher red blood cell distribution width (RDW) as for example in rheumatoid arthritis and SLE (Chikazawa et al., 2000; Caccavo et al., 2005) and inflammatory bowel diseases (Song et al., 2012). In these anemias of chronic disease (Weiss and Goodnough, 2005) erythropoiesis is not increased in proportion to the enhanced RBC clearance. No one in the medical field has considered the possibility that extra opsonization of aging RBC by IgG anti-lactoferrin may result in accelerated removal of normal, aging RBC and eventually in anemia. The presence of anti-lactoferrin in IgG eluates from ageing RBC of such patients would provide the proof.

The anemia developing from the enhanced clearance of aging RBC by bound anti-band 3 and anti-lactoferrin could be cured by treatment with human or bovine lactoferrin per os. Lactoferrin would complex anti-lactoferrin and thereby prevent it from binding to the 38 kDa fragment of band 3 on aging RBC. Indeed, treatment with lactoferrin appears to stop anemia of chronic disease: for example RBC from New Zealand Black mice, constitutively suffering from an autoimmune type RBC clearance, had fewer numbers of Coombs-positive RBC upon treatment with bovine lactoferrin (Zimecki et al., 1995). In humans 200 mg/day

of orally applied lactoferrin in combination with erythropoietin normalized the hemoglobin concentration in 75 cancer patients with anemia of chronic disease (Macciò et al., 2010). In fact, lactoferrin was as effective as injection of 125 mg ferric gluconate per week along with erythropoietin, but reduced serum ferritin significantly. Lactoferrin was also effective in treating pregnant women with iron deficiency anemia: Oral administration of lactoferrin (100 mg/twice a day for 30 days), that was iron-saturated to 30%, increased the total serum iron and hemoglobin concentrations to a greater extent than administration of ferrous sulphate (156 mg/day) in 60 anemic women (Paesano et al., 2009, 2010). These authors also found that the lactoferrin treatment lowered the serum concentration of IL6 from 32 to 12 pg/ml and therefore think that this mitigated inhibition of ferroportin 1 on macrophages and thereby enhanced iron export to blood for efficient erythropoiesis (Paesano et al., 2010). These thoughts call for clinical trials in which patients with anemia of chronic disease will be studied for IgG anti-lactoferrin antibodies in their serum and if positive, will eventually be treated with oral lactoferrin.

OTHER CLEARANCE MECHANISMS

All the above mentioned findings differ completely from the old idea that desialylation is the trigger for removal of senescent RBC (Henrich and Aminoff, 1983; Bratosin et al., 1995). Fudenberg and coworkers addressed this question from a different viewpoint and asked whether heat-eluted IgG from senescent RBC has the same specificity as anti-Thomson-Friedenreich antibodies (anti-T), that are generated against neuraminidase-treated RBC glycoproteins. A pretreatment of RBC with anti-T antibodies did not alter the binding of heat-eluted IgG (Khansari et al., 1983). Thus, the two binding sites differ from each other and IgG from senescent RBC do not bind at all to desialylated sites.

"Eryptosis or suicidal erythrocyte death" as portrayed by Lang et al. (2008) should not be considered a senescence-like mechanism in healthy humans. Eryptosis is induced by several types of stresses, in particular by entry of Ca^{2+}ions followed by the loss of potassium ions, cell shrinkage, exposure of exoplasmic phosphatidylserine, and recognition by phagocytes. Eryptosis is not a senescence variant, because RBC *in vivo* aged for 110–126 days do not expose PS as illustrated in chapter 2 (Franco et al., 2013). In fact Lang and coworkers have recently studied eryptosis on young and old human RBC and confirmed that PS exposure was similarly negligible in young and old RBC, but increased upon induction of eryptosis more so in old than young RBC (Ghashghaeinia et al., 2012). Eryptosis may contribute to accelerated RBC clearance in a number of systemic diseases like hemolytic uremic syndrome and in sepsis (Lang et al., 2008).

There were reports on yet another mechanism of how senescent RBC may be tagged. Galili and coworkers described anti-αGal antibodies as representing natural antibodies existing in the plasma of all humans at high concentrations of up to 1% of total IgG (Galili et al., 1986). Most of bound IgG could be stripped off from old RBC by galactose and RBC stripped from bound IgG were phagocytosed upon addition of anti-αGal. Clark and collaborators reinvestigated the role of anti-αGal and found that in eluates from senescent RBC 9–39% of total IgG had anti-αGal and 5–18% anti-band 3 specificity (Clark and Sorette, 1991). Considering the fact that human plasma contains 100–1000 times more anti-αGal than anti-band 3 NAbs, the retention of anti-αGal antibodies was due to incomplete removal by washing. A year later Galili and coworkers no longer reported that anti-αGal could be involved in removal of aged human RBC, because human cells lack the α1,3Gal structure, while this epitope has been found on surface glycoconjugates of other mammalian cells and on many bacterial surfaces (Hamadeh et al., 1992). In fact, anti-αGal is most likely an induced antibody rather than a NAb.

After Lutz had isolated and characterized anti-spectrin NAbs from human IgG, Wiener et al. found significantly increased plasma concentrations of anti-spectrin NAbs in splenectomized patients with β-thalassemia major and in patients with sickle cell anemia (Wiener et al., 1986). Surprisingly, eluates from patients' RBC also revealed anti-spectrin NAbs. The authors concluded that binding of these NAbs to intact RBC may either be due to abnormally exposed spectrin or because of a cross-reaction. Irrespective of the reason for bound anti-spectrin NAbs, the increased number of bound IgG as such most likely had a stimulating effect on the clearance of these cells. Berti and collaborators later reported analogous findings for RBC from rats and hypertransfused rats, incubated with induced rat anti-spectrin antibodies (Giuliani et al., 2000). In trying to understand the phenomenon the group of Lutz reported that an exoplasmic cross-linking of band 3 protein on human RBC not only enhanced binding of anti-band 3, but also that of purified ^{125}I-iodinated anti-spectrin NAbs 7–10 fold at 0°C in the presence of nearly physiological IgG and HSA concentrations (Hornig and Lutz, 2000). Binding of anti-spectrin NAbs was not competed by anti-band 3 NAbs and bound anti-spectrin NAbs even stimulated binding of anti-band 3 NAbs by 30%. While anti-spectrin NAbs bound at physiologic tonicity to band 3 or an associated protein by virtue of their inherent polyreactivity (Hornig and Lutz, 2000), their interaction with other components is increased at low ionic strength (Heegaard, 1993). The role of anti-spectrin NAbs is most likely to opsonize ghosts, generated from hemolysing RBC in circulation, as has been studied in detail (Salhany et al., 2001).

RBC contain a surface protein that protects RBC from being phagocytosed, CD47 (integrin-associated protein). When RBC in circulation bump into macrophages, CD47 actively prevents engulfment by binding to SIRPα on macrophages and this interaction provides a "do not eat me signal" [for a review see reference Matozaki et al. (2009)]. Recently, Burger et al. suggested that CD47 may act like a molecular switch from suppression to promotion of phagocytosis, when it may facilitate recognition and phagocytosis of aged RBC by macrophages (Burger et al., 2012). The authors think that RBC aging induces a conformational change in CD47 whereby it binds thrombospondin-1 and then interacts with SIRPα and induces phagocytosis of aged RBC. The evidence is weak, because the required concentration of the thrombospondin-1 peptide exceeded the thrombospondin-1 concentration in plasma by 10^3–10^4 (Lutz, 2013) and the phenomena had been studied on RBC that (a) were not actively leucocyte-depleted and (b) were oxidatively damaged by $CuSO_4$ and ascorbic acid rather than *in vivo* aged.

CONCLUDING REMARKS

Covered in this review are the mechanisms of senescence of RBC in healthy humans. These mechanisms may differ for RBC of patients with hereditary anemias or polycytemia and those observed in RBC during storage. In these cases calcium uptake, PS exposure and abnormal alterations in cell volume, membrane architecture, as well as redox balance may play a much more important role. The presentation of senescent RBC from healthy humans to phagocytes is mediated by complement, but initiated by naturally occurring anti-band 3 antibodies that bind to aggregated band 3 protein. A very similar, additive effect may have induced anti-lactoferrin antibodies in autoimmune diseases characterized by ANCA. The induced anti-lactoferrin antibodies bind to the carbohydrate portion of the band 3 protein and appear to accelerate clearance of otherwise normal senescent RBC and may induce anemia.

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First description of phosphofructokinase deficiency in spain: identification of a novel homozygous missense mutation in the PFKM gene

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Phosphofructokinase deficiency is a very rare autosomal recessive disorder, which belongs to group of rare inborn errors of metabolism called glycogen storage disease. Here we report on a new mutation in the phosphofructokinase (PFK) gene *PFKM* identified in a 65-years-old woman who suffered from lifelong intermittent muscle weakness and painful spasms of random occurrence, episodic dark urines, and slight haemolytic anemia. After ruling out the most common causes of chronic haemolytic anemia, the study of a panel of 24 enzyme activities showed a markedly decreased PFK activity in red blood cells (RBCs) from the patient. DNA sequence analysis of the *PFKM* gene subsequently revealed a novel homozygous mutation: c.926A>G; p.Asp309Gly. This mutation is predicted to severely affect enzyme catalysis thereby accounting for the observed enzyme deficiency. This case represents a prime example of classical PFK deficiency and is the first reported case of this very rare red blood cell disorder in Spain.

Keywords: phosphofructokinase deficiency, glycogen storage disease, PFKM gene, missense mutation, enzyme catalysis

INTRODUCTION

Phosphofructokinase (ATP: D- fructose-6-phosphate-1-phosphotransferase; EC 2.7.1.11; PFK) is a key regulatory enzyme of the glycolytic cycle and catalyses the conversion of fructose-6-phosphate to fructose-1,6-diphosphate (**Figure 1**). Human PFK is composed of three isoenzymes, muscle (M), liver (L), and platelet (P) (Vora, 1983; Nakajima et al., 2002). The P type is also known as Fibroblast type (F). Mammalian PFK is a tetrameric enzyme that is subjected to allosteric regulation. Tissue isozymes randomly aggregate to form homotetramers or heterotetramers depending on the relative abundance of the subunits in a particular tissue. PFK-M is the sole subunit in muscle cells whereas red blood cells (RBCs) contain both L and M subunits and form their hybrids (M4, L4, M3L, M2L2, and ML3).

Phosphofructokinase deficiency (OMIM 171 850) is a very rare autosomal recessive condition with heterogeneous clinical symptoms, mainly characterized by myopathy and/or haemolysis (Hirano and Di Mauro, 1999). Myopathy is caused by the accumulation of glycogen in muscle tissue due to the metabolic defect and is also known as glycogenosis type VII or Tarui disease. It is characterized by muscle pain, exercise-induced fatigue, cramps, and myoglobinuria. The observed clinical symptoms reflect lack of muscle PFK activity and partial reduction of enzymatic activity in erythrocytes. The latter usually is associated with mild haemolysis.

Up to now, only about 100 patients with PFK deficiency have been reported worldwide and 22 PFK-deficient *PFKM* alleles have been characterized. The gene encoding the M subunit (*PFKM*) has been assigned to chromosome 12q13.3 and spans 30 kb. It contains 24 exons and at least 3 promoter regions (Elson et al., 1990; Yamada et al., 2004). Among the detected mutations are mostly missense mutations and splicing defects.

We now describe here a Spanish patient with a clinical history of anemia, haemolysis, and intermittent muscle weakness, who was found to be homozygous for a novel mutation in the *PFKM* gene (c.926A>G). This mutation encodes the substitution of aspartic acid by glycine at residue 309 (p.Asp309Gly). This is the first description of PFK deficiency in Spain.

CASE REPORT

A 65-years-old woman with long standing hypertension and type 2 diabetes was referred to our Unit because of intolerance to exercise and chronic fatigue. From youth she suffered from spasms of random occurrence associated with muscle weakness, painful intolerance to small efforts, and intermittent dark urines, especially after exercise due to myoglobinuria, as revealed by urinalysis. Physical examination showed no weakness or muscle atrophy and only a moderate splenomegaly without hepatomegaly or lymphadenopathy. Parents were unavailable for study and there was no known consanguinity. The same clinical picture, however, was present in a non-smoker brother whereas the three patient's daughters were normal. Patient's clinical condition has remained stable in follow-up.

Complete blood count (CBC) showed moderate anemia (Hb: 115 g/L), with slight macrocytosis (105 fl) and increased

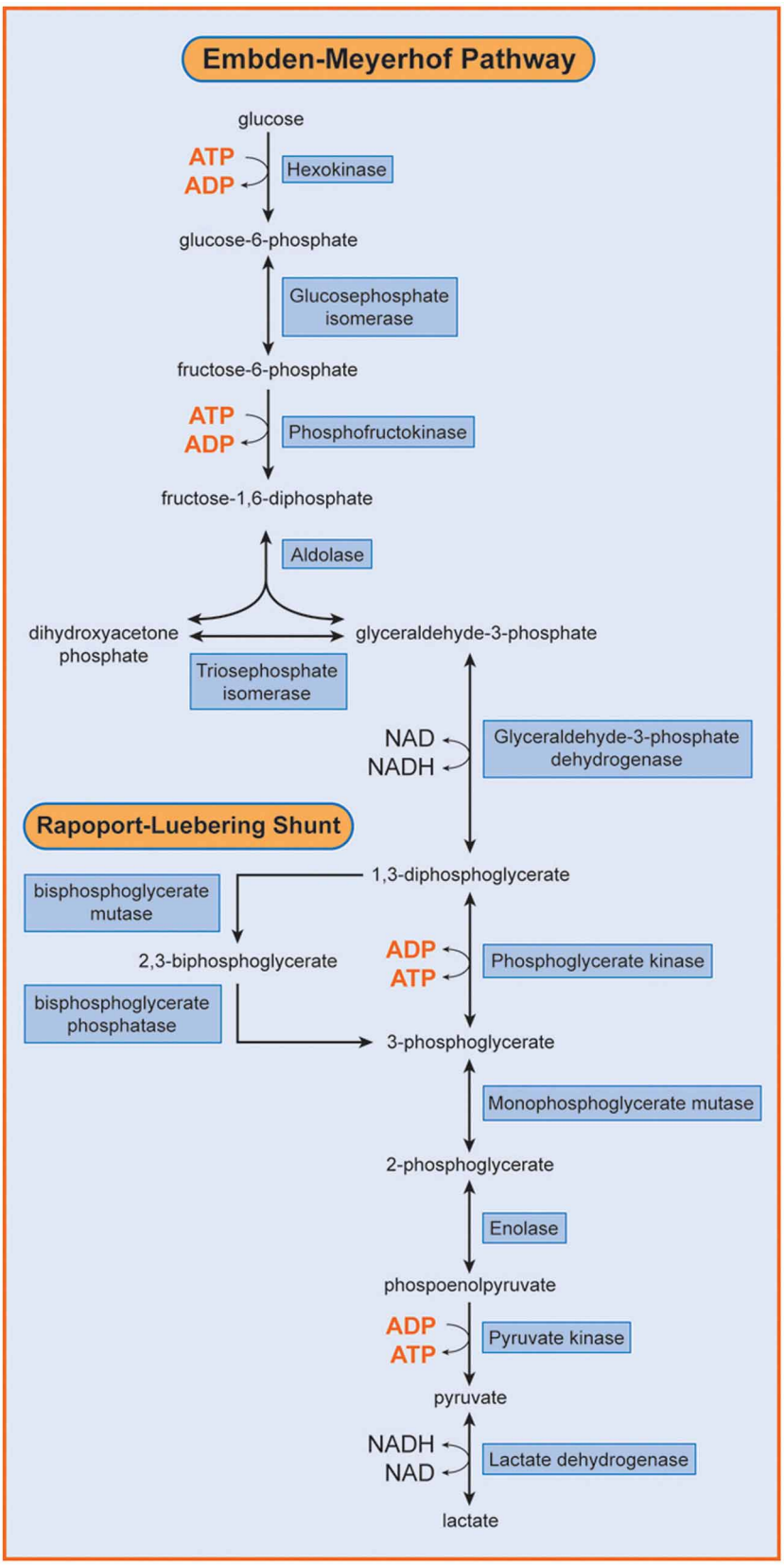

FIGURE 1 | Embden Meyerhof Pathway of RBC metabolism. Phosphofructokinase (PFK) catalyzes the transformation of fructose 6-phosphate into fructose 1,6 diphosphate. [Reproduced with permission from Van Wijk and van Solinge (2005)]

number of circulating reticulocytes (110×10^9/L). Leukocyte and platelet counts, as well as general serum biochemical analysis, were within normal range, except for a moderate rise in non-conjugated bilirubin, lactate dehydrogenase (LDH) and uric acid (hyperuricemia). Biological signs of diabetes mellitus type 2 were also present. The studies performed to rule out the origin of the anemia, discarded nutritional deficiencies (serum iron tests, cobalamin and serum folate were all normal), haemoglobinopathies (HPLC and thermal stability), and paroxysmal nocturnal haemoglobinuria (normal flow cytometry measurement of CD45 and CD49 in leukocytes and RBCs). Hereditary RBC membrane defects were ruled out by morphological observation of May-Grünwald-Giemas stained blood smears, and a normal osmotic fragility test. Extensive study of RBC enzyme activity measurements demonstrated a marked decrease (<30% of normal) in PFK activity (**Table 1**). DNA sequence analysis of individual exons of *PFKM*, including flanking splice sites, revealed that the patient was homozygous for a missense mutation in exon 11: c.929A>G. This mutation, that has not been previously reported in the literature, encodes the substitution of aspartic acid by glycine at residue 309 (p.Asp309Gly). Mutation prediction programs PolyPhen-2 (Adzhubei et al., 2010) and SIFT (Kumar et al., 2009) predict this mutation to be pathogenic (i.e., disease causing).

The complete lack of PFK activity in muscle was confirmed on both histological preparations and muscle extracts. Muscle abnormality was also confirmed by electromyography (EMG), that showed mild myopathic changes and by the forearm test, characterized by a plane lactate curve with normal increase of ammonium. As usual in patients with glycogenosis, a painful spasm occurred at the end of the forearm test. Muscle biopsy showed slight amounts of polysaccharide (PAS) not digested by diastase and abnormalities in NADH-TR reaction.

DISCUSSION

In this report, we describe, for the first time, the occurrence of PFK deficiency in Spain in an adult woman of 65 years of age. Her moderate haemolysis was associated with the accumulation of muscle glycogen comparable to that of Tarui disease. Muscle pain and exercise-induced fatigue and weakness associated with dark urines were the most relevant clinical manifestations. She was found to be homozygous for a novel missense mutation in *PFKM*, the gene that encodes the PFK-M subunit. On the amino acid level, this mutation (c.929A>G) causes the substitution of aspartic acid by glycine at residue 309. Asp309 is part of an α-helix, and replacement with glycine could disrupt this α-helix. Furthermore, structural analysis using the 3D molecular model of rabbit muscle type PFK (PDB entry 3O8L) showed that the p.Asp309Gly substitution is located in direct vicinity of the nucleotide (ADP) binding site in the center of the PFKM subunit (**Figure 2A**). PFK is allosterically activated by ADP, and the ADP binding site has been recently identified (Banaszak et al., 2011). Asp309 does not directly interact with ADP, however, substitution of Asp309 by glycine is likely to disrupt multiple hydrogen bonds with Gly177, Gly179, Ser 180, and Ser306 (**Figure 2B**). This loss of hydrogen bonds and the change of polarity and electrostatic interactions upon mutation of Asp309 will likely affect correct positioning of the side-chains of amino acids directly involved in ADP binding (**Figure 2B**), in particular neighboring residue Phe308, which makes stacking interaction with the adenine ring of ADP. This hypothesis is further supported by recent functional studies of the Asp543Ala change in PFKM. This mutation is associated with Tarui disease, and substitution of Asp543 by Ala was shown to disrupt hydrogen bonds with ADP (Brüser et al., 2012). We therefore postulate that decreased binding of the allosteric activator ADP will inhibit PFK enzymatic activity, in particular under low energy levels. These findings support the physiological importance of Asp390 for the recently identified ADP binding site.

Table 1 | Red blood cell enzyme activity measurements.

	Patient	Reference value
G6PD activity (IU/g Hb)	7.0	6.2–9.9
HK activity (IU/g Hb)	1.2	0.6–1.3
PGI activity (IU/g Hb)	61.9	46.0–66.0
PK activity (IU/g Hb)	15.3	9.5–15.6
PFK activity (IU/g Hb)	3.07	6.4–13.9

Glucose-6-phosphate dehydrogenase; HK, Hexokinase; PGI, Phosphoglucose isomerase; PK, Pyruvate kinase; PFK, Phosphofructokinase.

The missense mutation is thus predicted to lead to a less functional PFK-M subunit. In accordance with this, the complete lack of PFK activity in muscle was confirmed on both histological preparations and muscle extracts. The muscle abnormality was also confirmed by electromyography, ischemic exercise testing, histochemistry and electron microscopy. Furthermore, partial red blood cell PFK deficiency was reflected by the moderately decreased enzymatic activity in red blood cells, leading to mild haemolytic anemia

PFK is a key regulatory enzyme for glycolysis (Van Wijk and van Solinge, 2005) and catalyzes the irreversible transfer of phosphoryl from ATP to fructose-6-phosphate, and converts it to fructose-1,6-bisphosphate. Thus, tissues deficient in PFK cannot use free or glycogen-derived glucose as a fuel source and accumulate glycogen (glycogenosis). PFK deficiency (Tarui disease) was the first disorder recognized to directly affect glycolysis (Tarui et al., 1965). Since this first description of the disease, a wide range of biochemical, physiological and molecular studies have greatly contributed to our knowledge concerning not only PFK function in normal muscle, but also on the general control of glycolysis and glycogen metabolism. So far, more than one 100 patients have been described with prominent clinical symptoms characterized by muscle cramps, exercise intolerance, rhabdomyolysis and myoglobinuria, often associated with haemolytic anemia and hyperuricemia. In classic Tarui disease, the genetic defect involves the M isoform, resulting in the absence of enzymatic activity in the muscle. Erythrocytes lack the M4 and hybrid isozymes and only express the L4 homotetramers, resulting in about 50% of normal PFK activity (**Figure 3**). Thus, haemolysis is a result of partial erythrocyte PFK deficiency.

Despite PFK deficiency is a very rare autosomal recessive disease; its true incidence may be higher due to lack of recognition, as symptoms may be quite mild. In fact, our case highlights the latter, because the mild clinical presentation led her to be diagnosed,

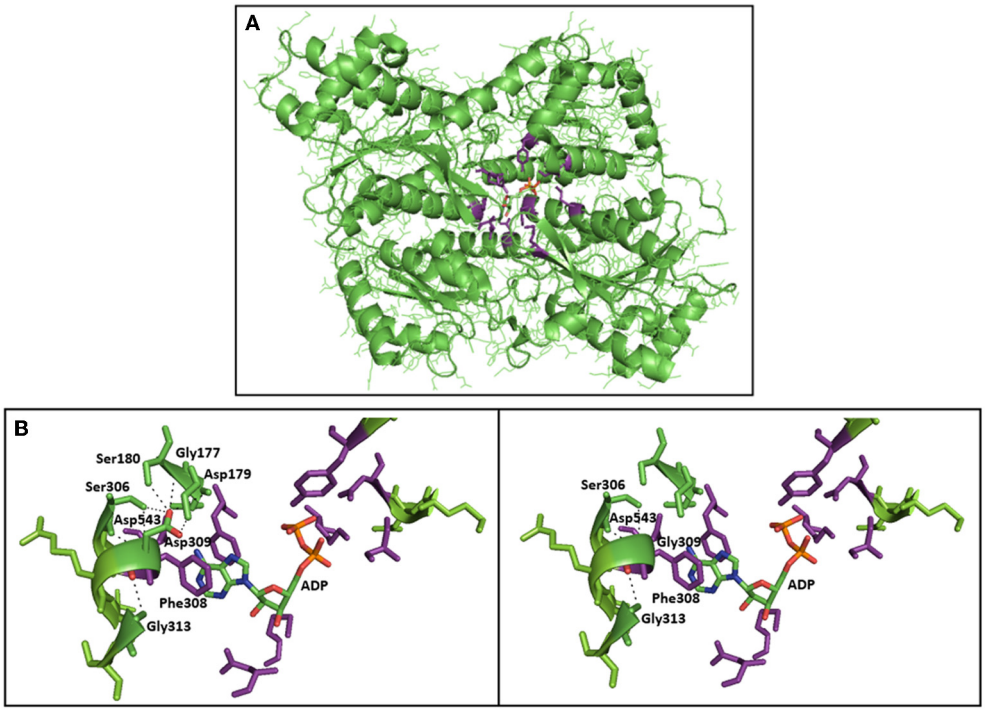

FIGURE 2 | 3D crystal structure of PFK subunit from rabbit skeletal muscle and 3D model of normal/mutated PFKM (PDB 3O8L—protein databank). (A) Allosteric nucleotide (ADP) binding site residues (purple) in the center of the rabbit subunit of PFKM, and the allosteric activator ADP are shown (atom coloring: carbon—green, nitrogen—blue, oxygen—red, phosphor—orange). **(B)** Close-up of the c.Asp309Gly mutation in PFKM. Asp309 is in close proximity of the ADP binding site. Asp309 does not directly interact with ADP. However, substitution of Asp309 by glycine is likely to disrupt multiple hydrogen bonds with Gly177, Gly179, Ser 180 and Ser306 (black dotted lines). This loss of hydrogen bonds, and the change of polarity and electrostatic interactions upon mutation of Asp309 will likely affect correct positioning of the side-chains of amino acids directly involved in ADP binding; in particular neighboring residue Phe308, which makes stacking interaction with the adenine ring of ADP.

for many years, as chronic fatigue, until the consideration of dark urines ultimately led to the study of chronic haemolysis. In our case the combination of RBC enzyme activity measurements and muscle biopsy analysis allowed for the correct diagnosis even at this late stage of life.

Generally, PFK deficiency presents in childhood. Clinical history however, defines 4 main subtypes: (1) classic, (2) infantile onset, (3) late onset, and (4) haemolytic. Most of the reported cases belong to the classic form, characterized by exercise intolerance, fatigue, muscle cramps with pain and myoglobinuria (Hirano and Di Mauro, 1999). A compensated haemolysis with jaundice, increased serum creatine kinase (CK) and hyperuricemia is also commonly present. Sometimes, nausea and vomiting appear after intense physical efforts Patients with the infantile onset may manifest as "floppy babies" that die within the first year of life. Symptoms include myopathy, psychomotor retardation, cataracts, joint contractures, and death during early childhood. They can also show evidence of arthrogryposis and mental retardation. Patients with the late-onset form may present in adulthood with progressive muscle weakness, cramps and myalgias in later life. Exercise ability, however, is low already in childhood, and a mild muscle weakness may appear in the 5th decade leading to severe disability. Diagnosis depends on patient history, physical examination and the findings from muscle biopsy, electromyography, ischemic forearm testing, CK testing. RBC enzyme activity measurement is, however, the easiest way to establish the definitive diagnosis. Fatal infantile type and late-onset forms of PFK clinical expression are very rare, with only several reported cases. The haemolytic form presents with hereditary non-spherocytic haemolytic anemia without muscle manifestations (Fujii and Miwa, 2000). Due to the molecular genetic heterogeneity, a clear-cut genotype-phenotype correlation has not been recognized in patients with PFK deficiency (Toscano and Musumeci, 2007). Unfortunately, no specific treatment or cure of enzyme deficiency exists. Although diet therapy may be highly effective at reducing clinical manifestations, Tarui disease resolves with rest. Fortunately, the condition does not progress to severe disability. Because the liver and kidneys express only the L isoform, these organs are spared.

About 100 cases of PFK deficiency have been reported in patients with Tarui disease from Europe, USA,and Japan with some predominance in Ashkenazi Jews (Fujii and Miwa, 2000). Up to know, 22 different mutations of PFK have been described. Missense and splicing mutations are the most frequently occurring mutations in *PFKM*. Intriguingly, PFK-deficient Ashkenazi Jews share 2 common mutations in the gene. A splicing defect caused by the G>A base change at the first nucleotide in intron

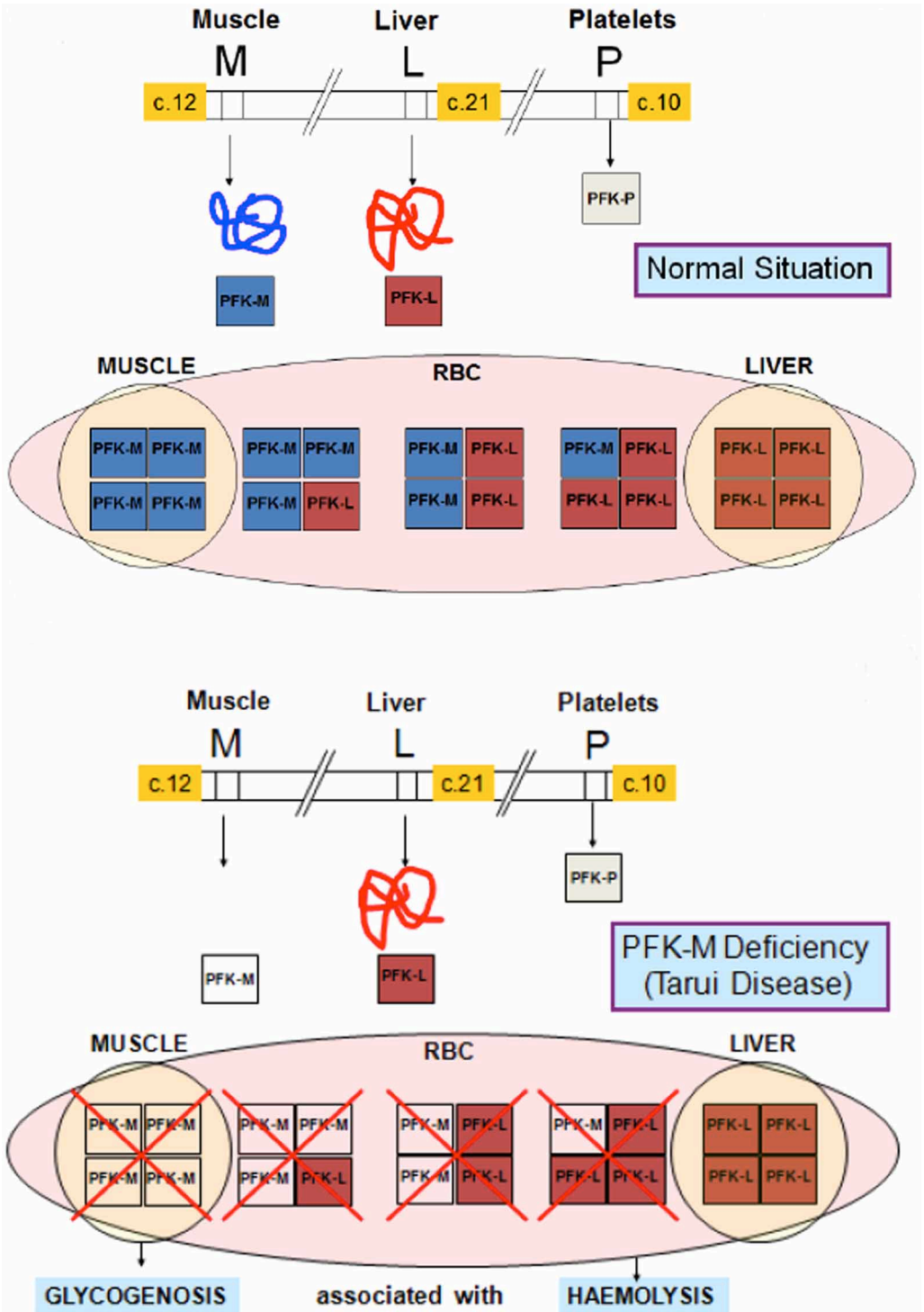

FIGURE 3 | Molecular basis of PFK deficiency. In classic Tarui disease, the genetic defect involves the M isoform of PFK enzyme, resulting a severe enzyme deficiency in muscle. Erythrocytes that normally have two homotetramers (M4 and L4) and three hybrid isozymes (M3L, M2L2 and ML3), lack the M4 isoform and the hybrid isozymes, and only express the L4 homotetramers, resulting in about 50% of normal PFK activity

5 (c.237+1G>A) accounts for 68% of mutant Ashkenazi alleles, and a single base deletion in exon 22 (c.2003delC) accounts for about 27% of mutant Ashkenazi alleles (Raben and Sherman, 1995). The here described homozygous patient is the first Spanish case described to be affected by this very rare disease. The identification of a novel homozygous missense mutation further extends the repertoire of PFK deficiency-associated mutations in *PFKM*.

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Calcium homeostasis in red blood cells of dialysis patients in dependence of erythropoietin treatment

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Previous studies provided evidence for a massively increased intracellular Ca^{2+} concentration in red blood cells (RBCs) of patients with end-stage renal disease (ESRD) (Paschen et al., 1971; Gafter et al., 1989), whereas the dialysis procedure itself led in average to an even slightly decreased RBC's Ca^{2+} content (Paschen et al., 1971; Długaszek et al., 2008). Based on a single cell approach we could qualitatively confirm these results (**Figure 1A**, 2 leftmost columns), although the extend of the Ca^{2+} increase was smaller compared to the cited investigations, which is presumably caused by differences in the methodology.

There is a good knowledge of Ca^{2+} related processes in RBCs (Bogdanova et al., 2013). Additionally, an increased intracellular free Ca^{2+} concentration in RBCs has been proposed as a trigger for intracellular aggregation (Andrews and Low, 1999; Kaestner and Bernhardt, 2002) as well as for endothelium-RBC adhesion (Hebbel et al., 1980; Mohandas and Evans, 1985) and experimental evidence has been provided (Noh et al., 2010; Steffen et al., 2011; Borst et al., 2012; Kaestner et al., 2012).

The molecular identity of Ca^{2+}- and non-selective cation channels in the RBC membrane is steadily increasing (Kaestner, 2011) and comprise of, e.g., the $Ca_V2.1$ (Andrews et al., 2002), the TRPC6 (Foller et al., 2008), the NMDA-receptor (Makhro et al., 2013), and the Piezo1 (Zarychanski et al., 2012).

The effect of erythropoietin (EPO) on RBCs ion homeostasis is controversially discussed. The group of Florian Lang found an inhibition of non-selective cation channels by EPO with a decreased number of eryptotic RBCs if patients were treated with EPO (Myssina et al., 2003). However, once Ca^{2+} entered the RBC, EPO has no beneficial effect toward the eryptotic symptoms caused by Ca^{2+} (Vota et al., 2013). In contrast to RBCs, hematopoietic progenitor cells display an increased cation-channel activity upon EPO exposure (Cheung et al., 1997; Tong et al., 2008). Because of its hematopoiesis stimulating properties, EPO became a widely used medication for treatment of anemic patients, including chronic renal disease, hematologic disorders, and acquired immune deficiency syndrome (Palmer et al., 2010; Goodnough and Shander, 2013). However, several studies highlighted the problem of an increased risk of thrombus formation, especially venous thromboembolism, in patients undergoing EPO therapy (Singbartl, 1994; Kliger et al., 2012; Goodnough and Shander, 2013).

Therefore we investigated the free Ca^{2+} concentration in RBCs from ESRD (dialysis) patients under EPO treatment. Blood samples from healthy donors, ESRD patients and EPO treated ESRD patients were analyzed by fluorescence live cell imaging as previously described (Wang et al., 2013) (**Figure 1**). As depicted in **Figure 1A**, at rest, RBCs from ESRD patients show higher Ca^{2+} concentration compared with healthy donors, while EPO treatment let to a slightly decreased free internal Ca^{2+} concentration, indicating an inhibition of constitutively active channels in resting RBCs. Although the histograms (**Figure 1Ab**) give an impression of the distribution, the method lacks quantitative information concerning the Ca^{2+} concentration (Kaestner et al., 2006). However, when compared to control conditions, the width of the distribution of Ca^{2+} content is wider in ESRD patients or EPO-treated ESRD patients, leading to the conclusion that the cellular heterogeneity is greater in patients than in healthy subjects. In a further step we investigated the Ca^{2+} influx in RBC from healthy donors and ESRD patients after hormonal stimulation in dependence of EPO treatment (**Figure 1B**). As a stimulation substance we selected prostaglandin E_2 (PGE_2), which is released from activated platelets (Smith et al., 1973) but can also be released from RBCs themselves when they pass small capillaries (Oonishi et al., 1998). The curves in **Figure 1Ba** present typical example traces for Ca^{2+} curves in RBCs, while **Figure 1Bb** shows the statistical analysis. Healthy patients show an increase in Ca^{2+} after PGE_2 stimulation as we have previously shown (Kaestner et al., 2004). EPO treatment prevents Ca^{2+} entry resulting in Ca^{2+} levels below control conditions confirming the results of the Lang group (Myssina et al., 2003). However, pretreatment with EPO even suppressed the Ca^{2+} entry provoked by PGE_2. In ESRD patients, PGE_2 stimulation leads to a Ca^{2+} increase, which is significantly higher than in RBCs of healthy donors. In EPO treated ESRD patients the PGE_2 induced Ca^{2+} increase was significantly suppressed compared to RBCs of

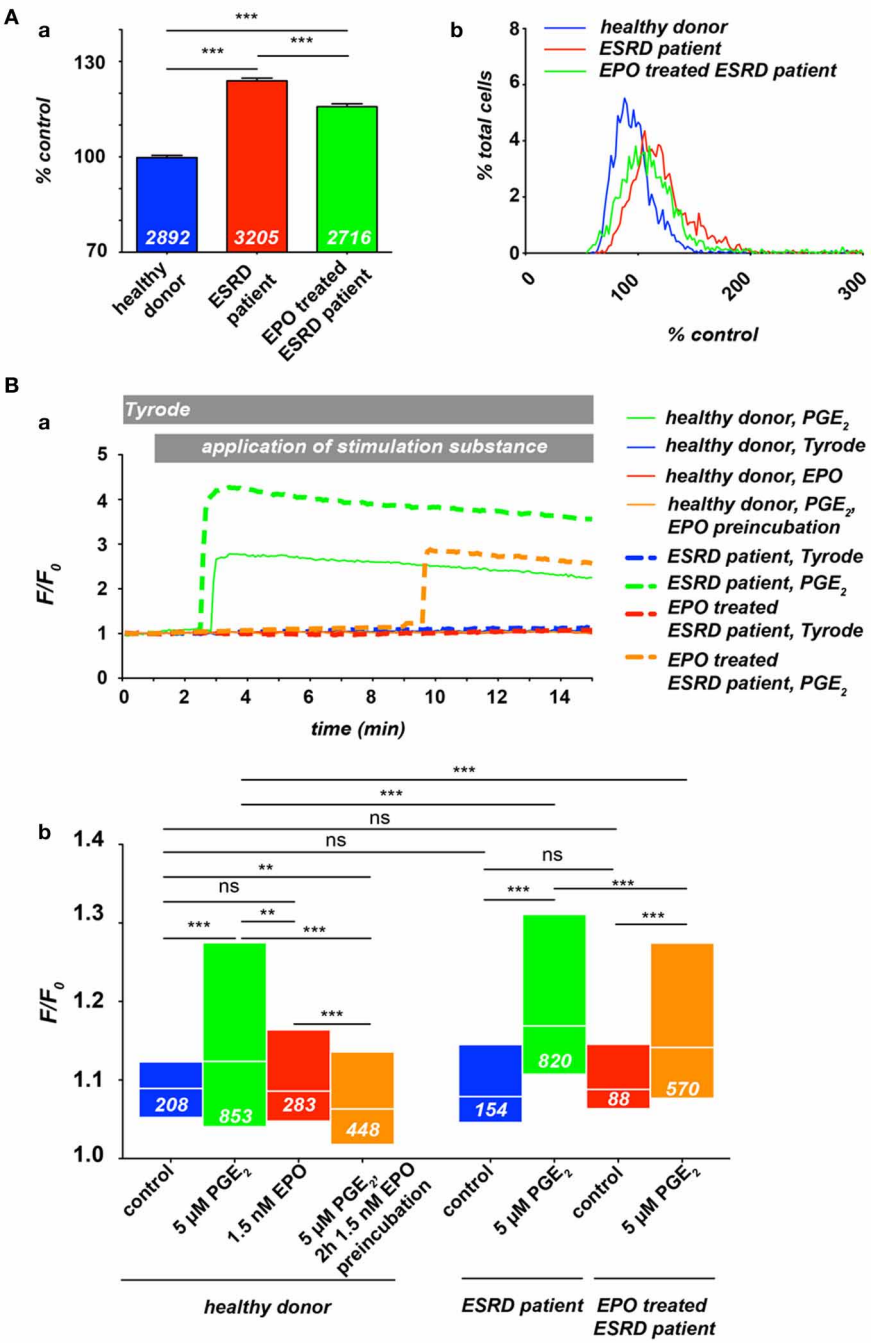

FIGURE 1 | Ca^{2+} in RBCs of healthy donors and end-stage renal disease (ESRD) patients with a renal anaemia. If patients were under EPO treatment, the last EPO administration was within 5 days of blood sampling. All experimental procedures are previously described in detail (Wang et al., 2013). **(A)** Resting Ca^{2+} in terms of relative Fluo-4 fluorescence intensity of RBCs suspended in plasma. **(Aa)** Statistical analysis of RBC Ca^{2+} of at least 3 individuals in each group. The white numbers indicate the number of cells analyzed and the error bars represent standard error of mean. **(Ab)** Histograms of the relative fluorescence intensity distribution of the measurements presented in **(Aa)**. **(B)** Intracellular Ca^{2+}-traces in RBCs under different conditions: control (Tyrode solution containing in mM: 135 NaCl, 5.4 KCl, 10 glucose, 1 $MgCl_2$, 1.8 $CaCl_2$ and 10 HEPES; the pH was adjusted to 7.35 using NaOH) or stimulation with 5 µM PGE_2 and/or 1.5 nM EPO. **(Ba)** Typical example traces of Fluo-4 fluorescence intensity over time for all conditions tested. Traces were chosen due to their typical intensity of response, which are analyzed in **(Bb)**. Differences in the onset of the response were equally distributed over a wide range for all conditions tested (data not shown). However, it is worthwhile to mention that in case of stimulation only a limited number of cells are responders (Kaestner et al., 2004), which explains the difference in normalized fluorescence intensity (F/F_o) between the example traces and the statistical analysis of the entire cell population measured. The white numbers indicate the number of cells analyzed. For the statistical evaluation a Mann–Whitney test was performed and the following convention was used: $^{***}p < 0.001$, $^{**}p < 0.01$, and ns $p > 0.05$.

non-treated patients even in the putative absence of EPO during the experiment.

The results of **Figure 1** suggest that the clinically observed thrombotic complications in patients treated with EPO seem not to be primarily caused by an elevated Ca^{2+} content of RBCs of these patients. However, it is hypothesized that EPO causes thrombosis as a result of inflammation (Tobu et al., 2004). The elevated basal Ca^{2+} level in RBCs of ESRD patients (Paschen et al., 1971; Gafter et al., 1989; **Figure 1A**) may enhance a blood clotting once initiated by an inflammation processes. It is a substantial finding that the response of RBC to hormonal stimulation or other treatments, like the dialysis itself, in terms of Ca^{2+} entry shows a wide variation between RBC within a population, but also between different individuals (Paschen et al., 1971; Wang et al., 2013; **Figure 1Ba**).

In light of the above statements concerning the thrombotic events and the molecular players, the perspective of medical treatment must be based on a personalized diagnosis followed by a personalized medication. This applies to ESRD patients as well as for treatments of other anemias. Techniques and procedures allowing such an individualized approach presumably based on the combination of RBC population measurements and single cell techniques (Minetti et al., 2013) need urgently to be developed.

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Primitive and definitive erythropoiesis in mammals

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Red blood cells (RBCs), which constitute the most abundant cell type in the body, come in two distinct flavors- primitive and definitive. Definitive RBCs in mammals circulate as smaller, anucleate cells during fetal and postnatal life, while primitive RBCs circulate transiently in the early embryo as large, nucleated cells before ultimately enucleating. Both cell types are formed from lineage-committed progenitors that generate a series of morphologically identifiable precursors that enucleate to form mature RBCs. While definitive erythroid precursors mature extravascularly in the fetal liver and postnatal marrow in association with macrophage cells, primitive erythroid precursors mature as a semi-synchronous cohort in the embryonic bloodstream. While the cytoskeletal network is critical for the maintenance of cell shape and the deformability of definitive RBCs, little is known about the components and function of the cytoskeleton in primitive erythroblasts. Erythropoietin (EPO) is a critical regulator of late-stage definitive, but not primitive, erythroid progenitor survival. However, recent studies indicate that EPO regulates multiple aspects of terminal maturation of primitive murine and human erythroid precursors, including cell survival, proliferation, and the rate of terminal maturation. Primitive and definitive erythropoiesis share central transcriptional regulators, including Gata1 and Klf1, but are also characterized by the differential expression and function of other regulators, including myb, Sox6, and Bcl11A. Flow cytometry-based methodologies, developed to purify murine and human stage-specific erythroid precursors, have enabled comparative global gene expression studies and are providing new insights into the biology of erythroid maturation.

Keywords: primitive erythropoiesis, definitive erythropoiesis, yolk sac, globin, cytoskeleton

INTRODUCTION

RBCs comprise the most abundant cell type in the body and function primarily to transport oxygen and carbon dioxide. More than a century ago it was recognized that the mature RBCs in the bloodstream of mammals lack a nucleus, while the RBCs of adult birds, amphibians, and fish retain a nucleus throughout their lifespan in the circulation (Gulliver, 1875). Examination of the bloodstream of developing mammalian embryos at the beginning of the last century revealed the presence of two distinct, temporally overlapping populations of erythroid cells. The earliest population consisted of larger, nucleated cells and the subsequent population consisted of smaller, enucleated RBCs (Maximow, 1909). The transient presence of the larger, nucleated erythroid cells during early development was termed "primitive" erythropoiesis to distinguish it from the "definitive" form of erythropoiesis that occurred not only during late fetal life but throughout all of postnatal life. This review provides an overview of primitive and definitive erythropoiesis and relies primarily on studies of the mouse as a model of mammalian biology where these distinct erythroid lineages have been most intensively studied.

DEFINITIVE ERYTHROPOIESIS

Definitive erythropoiesis occurs in the fetal liver and postnatal bone marrow and is characterized by the movement of lineage-committed cells through progenitor, precursor, and mature RBC compartments (**Figure 1**). The progenitor and precursor compartments occur in protected extravascular spaces and are associated with cellular amplification and maturation. These compartments sustain the third, functional compartment, which consists of RBCs circulating within the vascular network.

DEFINITIVE ERYTHROID PROGENITORS

The most immature erythroid compartment consists of lineage-committed, definitive erythroid progenitors, termed burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E). These progenitors are defined by their ability to form colonies of mature erythroid cells in semisolid media. BFU-E-derived colonies require 7 and 14 days in mouse and human systems, respectively, to form mature colonies typically containing more than a thousand erythroid cells. In contrast, the more mature CFU-E progenitors require only 2 and 7 days in mouse and human systems, respectively, to form mature colonies that consist of only 16–32 cells. Thus, CFU-E are only 4–5 cell divisions upstream of mature RBCs.

The cytokine erythropoietin (EPO) is necessary for the ability of CFU-E to generate colonies *in vitro*. CFU-E are exquisitely dependent on EPO for their survival (Koury and Bondurant, 1990). EPO levels in the bloodstream, regulated by hypoxia, are thought to modulate the number of CFU-E in the marrow and thus regulate the output of definitive RBCs (Koury and Bondurant, 1992). EPO acts through its specific receptor, EPOR, characterized by a single transmembrane domain that dimerizes upon ligand activation. Downstream signals occur

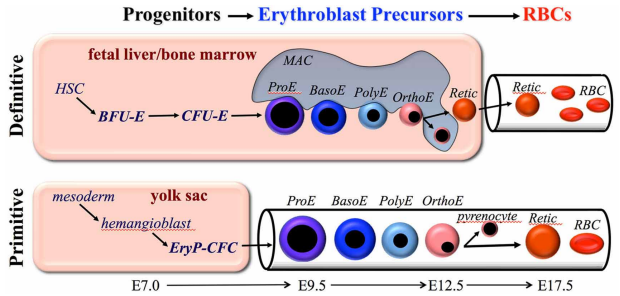

FIGURE 1 | Overview of primitive and definitive erythropoiesis. Both forms of erythroid cell production are characterized by the progressive movement of cells through three compartments: progenitors, erythroblast precursors, and red blood cells (RBCs). Erythroid progenitors (BFU-E, CFU-E, and EryP-CFC) are defined by their capacity to form colonies of maturing erythroid cells *in vitro*. Erythroid precursors are defined morphologically as proerythroblasts (ProE), basophilic erythroblasts (Baso), polychromatophilic erythroblasts (PolyE), and orthochromatic erythroblasts (OrthoE). OrthoE enucleate to form a pyrenocyte, that contains the condensed nucleus, and a reticulocyte (Retic), that goes on to mature into a RBC. Definitive erythropoiesis in the adult organism is derived from hematopoietic stem cells (HSC), while primitive erythropoiesis occurs just once from mesoderm cells in the early embryo.

through Jak2/Stat5, PI3K/AKT, and MAPK pathways (reviewed by Richmond et al., 2005). The importance of EPO signaling through its cognate receptor was demonstrated by the identical phenotype of EPO-null and EPOR-null mice: fetal death at embryonic day 13.5 (E13.5) associated with severe anemia and the complete lack of definitive RBCs (Wu et al., 1995; Kieran et al., 1996; Lin et al., 1996). The restoration of EPOR specifically in erythroid lineages rescued the lethal phenotype, indicating that anemia is indeed the cause of *in utero* death (Suzuki et al., 2002). Recent studies provide evidence that BFU-E and CFU-E can undergo limited self-renewal, particularly in response to acute stress such as anemia and that the self-renewal of CFU-E is dependent on the action of EPO (Flygare et al., 2011; Peslak et al., 2012).

DEFINITIVE ERYTHROID PRECURSORS

The second erythroid compartment consists of morphologically identifiable, nucleated precursors that progress from proerythroblast (ProE) to basophilic (BasoE), polychromatophilic (PolyE), and orthochromatic (OrthoE) forms (**Figure 1**). Erythroid precursor maturation is characterized by progressive (1) erythroblast expansion through a limited set of symmetric cell divisions, (2) accumulation of hemoglobin, (3) decrease in cell size, (4) nuclear pyknosis, and (5) decrease in RNA content. The morphologic criteria distinguishing these cells has relied primarily on the progressive nuclear condensation combined with changes in cytoplasmic staining, which reflects the degree of hemoglobin and RNA content (**Figure 1**). Enhanced flow cytometric approaches to distinguish these subpopulations have been developed for both mouse and human systems and have provided the capacity to analyze large numbers of cells and to physically isolate these precursors for further study (Koulnis et al., 2011; Hu et al., 2013; Kingsley et al., 2013; Liu et al., 2013). In addition, an imaging flow cytometry-based approach, which weds immunophenotypic analyses with characterization of morphologic features, has been developed to quantify multiple cellular aspects associated with progressive erythroid maturation, including their enucleation (McGrath et al., 2008a; Peslak et al., 2011, 2012; Konstantinidis et al., 2012).

Definitive erythroblasts mature in the fetal liver and postnatal bone marrow within erythroblastic islands, composed of erythroblasts physically attached to central macrophage cells (reviewed by Chasis and Mohandas, 2008). Macrophages have been shown to promote erythroblast proliferation, particularly in the context of stress erythropoiesis (Hanspal et al., 1998; Rhodes et al., 2008; Chow et al., 2013; Ramos et al., 2013). In mammals, the end result of precursor maturation is enucleation, which results in the formation of two cell types. The first population consists of reticulocytes that contain most of the cytoplasm and hemoglobin, as well as the proteins needed to form a unique cytoskeletal network (Gaiduschek and Singer, 1979; Koury et al., 1989; Lee et al., 2004; Liu et al., 2011). The second population consists of pyrenocytes ("extruded nuclei") that contain the condensed nucleus surrounded by a lipid bilayer and thin rim of cytoplasm (McGrath et al., 2008a). Enucleation is a complex process that involves multiple steps including the establishment of cell polarity through microtubule action, the formation of a contractile actomyosin ring, vesicle formation, and coalescence of lipid rafts between reticulocyte and pyrenocytes (Keerthivasan et al., 2010; Konstantinidis et al., 2012; Wang et al., 2012). Soon after their formation in the fetal liver and bone marrow, pyrenocytes flip phosphotidylserine onto their cell surface, providing an "eat me" signal, and are rapidly ingested by macrophages (Yoshida et al., 2005).

Reticulocyte maturation is a complex process that results in an approximately 20% loss of plasma membrane surface area, reduced cell volume, increased association of the cytoskeleton to the outer plasma membrane, and the loss of all residual cytoplasmic organelles, including mitochondria and ribosomes (Johnstone, 1992; Waugh et al., 2001). Organelle clearance occurs through both autophagy and exocytosis (Griffiths et al., 2012). The membrane changes convert a multilobulated immature reticulocyte into a biconcave disc with improved viscoelasticity. All of these changes prepare the reticulocyte for its rigorous sojourn of 45 and 120 days in the bloodstream of adult mice and humans, respectively.

DEFINITIVE RBCs

The third erythroid compartment consists of circulating reticulocytes and mature RBCs (**Figure 1**). Steady-state levels of RBCs are maintained by the continuous production and release of reticulocytes into the bloodstream to balance the removal of senescent RBCs by macrophage cells, which are localized primarily in the spleen (Bennett and Kay, 1981). In adult humans, this steady-state production results in the egress of more than 2 million reticulocytes every second into the bloodstream. This incredible number is estimated as follows: adult humans normally contain approximately 5×10^6 RBCs per microliter of blood and the blood volume of a 70 kg person is approximately 5 l, which amounts to 2.5×10^{13} total circulating RBCs. Since the lifespan of human

RBCs is approximately 115 days (Franco, 2012), we replace on average 1/115th of our red cells every day, or 2.2×10^{11} RBC/day, which amounts to 2.5×10^{6} RBCs/s.

The membrane of mature definitive RBCs is composed of a cholesterol/phospholipid plasma membrane to which is anchored an elastic network of spectrin-based cytoskeletal proteins containing actin and protein 4.1 at their junctions (reviewed by Mohandas and Gallagher, 2008). The anchoring of the cytoskeleton to the lipid bilayer occurs at these junctions through glycophorin C. In addition, interactions through ankyrin-band 3 stabilize the cytoskeleton to the lipid bilayer. This unique cytoskeletal network ensures that mature RBCs can passively deform during their repeated passage through capillary networks, yet ultimately maintain their biconcave shape. All of the components of the cytoskeleton accumulate during erythroblast maturation (Chen et al., 2009). Abnormalities of cytoskeletal proteins lead to hemolytic disorders in children and adults, including hereditary spherocytosis and elliptocytosis (Gallagher, 2013).

As RBCs age, surface area and volume, but not hemoglobin, are progressively lost resulting in increased RBC density. Senescent RBCs are cleared by splenic macrophages that recognize, among other changes, clustered band 3 on the RBC surface (Low et al., 1985). In addition, CD47 interactions with thrombospondin-1 on senescent RBCs can convert "don't eat me" to "eat me" signals leading to RBC clearance by macrophage cells (Burger et al., 2012).

PRIMITIVE ERYTHROPOIESIS

PRIMITIVE ERYTHROPOIESIS- EMERGENCE IN THE YOLK SAC FROM A TRANSIENT WAVE OF PROGENITORS

The first blood cells in the mammalian conceptus emerge in "blood islands" within the yolk sac. These pools of primitive erythroblasts differentiate from mesoderm cells soon after the onset of gastrulation. Examination of carefully staged mouse embryos revealed the presence of a unique erythroid progenitor, termed EryP-CFC (Wong et al., 1986; Palis et al., 1999). This transient wave of EryP-CFC emerges in the yolk sac at E7.25, peaks in numbers at E8.25, and are no longer detectable at E9.0 (Palis et al., 1999). EryP-CFC, like their definitive erythroid progenitor counterparts, require the addition of EPO for *in vitro* colony formation. Murine EryP-CFC require 5 days of *in vitro* culture to form colonies containing several hundred mature primitive erythroid cells. Thus, EryP-CFC contain a proliferative potential that is intermediate to that of the definitive BFU-E and CFU-E progenitors. In addition, primitive erythroid progenitors, unlike definitive erythroid progenitors, are incapable of even limited self-renewal when cultured *ex vivo* in the presence of EPO, SCF and dexamethasone, a difference associated with the differential expression of the receptors for the latter two factors (England et al., 2011).

A global gene expression study of E7.5 and E8.5 primitive erythroid cells reveals a gene profile characteristic of high aerobic glycolysis, suggesting that these progenitors share the "Warburg effect" with many cancer cell types that are found in hypoxic environments (Isern et al., 2011). Consistent with a role for hypoxia signaling in EryP-CFC, increases in progenitor numbers and in colony size were detected when yolk sac cells were cultured in low oxygen compared with room air. Interestingly, low oxygen conditions facilitated the detection of BFU-E in early mouse embryos, suggesting that hypoxia signaling also plays a role in the emergence of definitive erythroid progenitors in the yolk sac (Borges et al., 2012).

PRIMITIVE ERYTHROPOIESIS- TERMINAL PRECURSOR MATURATION

Examination of circulating blood cells over developmental time in rodent embryos revealed that primitive erythroblasts undergo progressive morphological maturation (Morioka and Minamikawa-Tachino, 1993; Kingsley et al., 2004; Fraser et al., 2007). Consistent with their origin from a transient wave of yolk sac-derived EryP-CFC at E7.5–E8.5, a semi-synchronous wave of maturing nucleated primitive erythroid precursors is evident in the mouse embryo between E9.5–E12.5 (**Figure 1**). Primitive erythroid precursors mature with progressive characteristics similar to their definitive counterparts, including (1) expansion of erythroblast numbers through a limited set of symmetric cell divisions, (2) accumulation of hemoglobin, (3) decrease in cell size, (4) nuclear pyknosis, and (5) decrease in RNA content. In addition, primitive erythroblasts lose the intermediate filament vimentin and nuclear histone proteins, changes that also occur during definitive erythroblast maturation (Sangiorgi et al., 1990; Morioka et al., 1998). By E12.5, the primitive erythroblasts have matured to an orthochromatic stage and cell division ceases.

The unique expression in primitive erythroid cells of embryonic globin genes both from the beta and from the alpha globin clusters has facilitated their identification and study, even after definitive cells have begun to emerge in the fetus. From the beta globin cluster, the embryonic εy- and βH1-globin genes are expressed in the mouse, and the ε- and γ-globin genes are expressed in human (**Table 1**). In contrast, the "adult" β1- and β2-globin genes are expressed in the mouse, and "fetal" γ- and "adult" β-globin genes are expressed in human definitive erythroid cells. From the alpha globin cluster in both mouse and human, primitive cells also express the embryonic ζ- and α-globin genes. The change in the expression of embryonic, fetal (for human), and adult globin genes during development has been termed "switching" and has been extensively studied over the past several decades with the ultimate goal of treating children and adults with hemoglobinopathies (reviewed by Stamatoyannopoulos, 2005; Sankaran et al., 2010).

Table 1 | Expression of globin genes and transcriptional regulators in primitive and definitive murine erythroid cells.

	Primitive erythropoiesis	Definitive erythropoiesis
Globin gene expression	ζ and α, βH1 and εy >> β1 and β2	α, almost exclusively β1 and β2
Transcriptional regulators	Gata1, Klf1, Tal1, Lmo2, Ldb1	Gata1, Klf1, Tal1, Lmo2, Ldb1, Myb, Sox6, Bcl11a

The transcriptional regulators Sox6 and Bcl11A have been identified as important regulators of globin gene expression, since they are expressed in definitive, but not primitive, erythroid cells, where they act to suppress the expression of embryonic globin genes (Yi et al., 2006; Sankaran et al., 2009). Erythroid cells can also switch their hemoglobin expression as they mature. This has been best characterized in primary mouse primitive erythroblasts, which undergo βH1- to εy-globin and ζ- to α-globin "maturational" globin switches (Kingsley et al., 2006).

For more than a century it was thought that primitive erythroblasts remain nucleated throughout their lifespan in the fetal circulation. However, large, enucleated "megalocytes" having the same hemoglobin content as nucleated yolk sac erythroblasts were detected at late stages of gestation in the mouse embryo (Bethlenfalvay and Block, 1970; Steiner and Vogel, 1973). Using antibodies specific for embryonic globins to unequivocally identify primitive erythroid cells, it was determined that primitive erythroblasts in the mouse fetus enucleate between E12.5–E16.5 of gestation (Kingsley et al., 2004). These findings have been confirmed in mice containing a GFP transgene driven by the human ε-globin promoter to identify primitive erythroid cells (Fraser et al., 2007). A transient population of primitive pyrenocytes was also detected during this time period in the fetal circulation of mice (McGrath et al., 2008b). Importantly, the total number of primitive erythroid cells does not decrease between E12.5 and E16.5, consistent with the cessation of cell division in late-stage primitive erythroblasts and the enucleation of the entire population of primitive erythroid cells (Kingsley et al., 2004).

PRIMITIVE RBCs

Reticulocyte maturation is associated with a significant loss of surface area and volume associated with remodeling of the cytoskeleton. Extremely little is known about the components of membrane cytoskeleton of primitive erythroid cells. The expression of band 3 and glycophorin A are highly upregulated as early as E8.5 (Isern et al., 2011). Spectrin and ankyrin transcripts have been identified in E10.5–E12.5 primitive erythroblasts (Peters et al., 1992). Since primitive erythroblasts mature in the circulation, they may assemble a functional cytoskeleton prior to their enucleation and terminal maturation into RBCs. However, it is not known when during their maturation the cytoskeleton is assembled. We have recently examined the biomechanical properties of murine primitive erythroid cells during the developmental time they undergo enucleation, i.e., between E12.5 and E17.5 (Waugh et al., 2013). Late-stage primitive erythroblasts have membrane deformability similar to mature definitive RBCs, a finding consistent with their need to survive the stresses of fetal circulation. As primitive erythroid cells enucleate between E12.5 and E17.5, the physical association of their outer membrane bilayer with the underlying cytoskeleton increases. Primitive erythroid cells also lose 35% of their surface area and 50% of their volume between E14.5 and E17.5. Interestingly, the loss of surface area and volume occurs whether or not the cells are enucleated (Waugh et al., 2013). These data suggest that, unlike definitive erythropoiesis, the maturational processes of membrane remodeling and enucleation are uncoupled in terminally maturing primitive erythroid cells.

While the lifespan of primitive RBCs is not known, primitive RBCs have been detected for several days after birth (Kingsley et al., 2004; Fraser et al., 2007). These data indicate that primitive RBCs can circulate for at least 5–7 days after they enucleate. It is not known where or by what mechanisms senescent primitive RBCs are recognized and cleared from the vasculature.

THE ROLE OF EPO IN PRIMITIVE ERYTHROPOIESIS

The role of EPO in primitive erythropoiesis has been surrounded by controversy. Early studies showed that EPO failed to increase heme synthesis in cultures of whole mouse embryos containing primitive erythroid cells (Cole and Paul, 1966), but the same group subsequently reported increased heme synthesis when EPO was added to cultures of disaggregated embryonic cells (Bateman and Cole, 1971). EPOR transcript expression has been identified in yolk sac blood islands between E7.5–E8.5 and in the yolk sac of E9.6–11.5 mouse embryos (McGann et al., 1997; Makita et al., 2001), while EPOR protein has been quantified on the cell surface of maturing primitive erythroblasts in the fetal hamster (Boussios et al., 1988). Furthermore, EPO increased erythroid cell numbers and embryonic globin expression in E7.5 yolk sac explants (Palis et al., 1995). Targeted disruption of EPOR causes a marked reduction of primitive erythroblasts by E10.5–E11.5 and a profound anemia by E12.5 (Wu et al., 1995; Kieran et al., 1996; Lin et al., 1996; Malik et al., 2013). Recently, a more detailed analysis of primitive erythropoiesis in EPOR-null mouse embryos has revealed important functions of EPOR signaling in terminal erythropoiesis, including reduction of primitive erythroblast proliferation associated with increased p27 expression, advanced cellular maturation, and markedly elevated rates of apoptosis associated with an imbalance in pro- and anti-apoptotic gene expression (Malik et al., 2013). Little EPO is expressed in the early mouse yolk sac (Malik et al., 2013), however, neuroepithelial cells of E8.5–E11.5 mouse embryos have recently been shown to express EPO and likely serve to support the early maturation of primitive erythroid precursors (Suzuki et al., 2013).

HUMAN PRIMITIVE ERYTHROPOIESIS

While primitive erythropoiesis has been most thoroughly investigated in the mouse model, relatively little is known about primitive erythropoiesis in humans owing both to ethical concerns and to the physical inaccessibility of the early embryo. Blood islands filled with primitive erythroblasts first arise at 18–20 days of gestation (Bloom and Bartelmez, 1940; Luckett, 1978; Kelemen et al., 1979). Primitive erythroblasts are the only circulating erythroid cells in human embryos from 3 to 6 weeks of gestation and nucleated primitive erythroid cells have been identified in the fetal circulation of the human embryo throughout the first trimester (Knoll, 1927). Human primitive erythroblasts can physically interact with macrophages within the placenta and, like their murine counterparts, also enucleate *in vivo* (Van Handel et al., 2010).

Human primitive erythroblasts express the embryonic ε- and ζ-globin genes. A maturational ζ- to α-globin switch occurs between 5 and 7 weeks of gestation in primitive erythroid cells (Peschle et al., 1985). Primitive erythroid cells derived from the *in vitro* differentiation of human ES cells can serve as a surrogate

model system for early stages of human development. Studies of embryonic stem cell-derived human primitive erythroblasts indicate that they, like their primary counterparts, undergo a "maturational" switch from hemoglobin Gower I ($\zeta_2\varepsilon_2$) to hemoglobin Gower II ($\alpha_2\varepsilon_2$) (Qiu et al., 2008). Recently, primitive erythroblasts derived from human ES have been shown to require EPO for their terminal maturation. Similar to primitive erythropoiesis in EPOR-null embryos, human primitive erythroblasts deprived of EPO undergo increased apoptosis and accelerated maturation at late maturational stages (Malik et al., 2013). These studies indicate that the role of EPO in terminal maturation of primitive erythroid precursors is evolutionarily conserved in mammals.

Little is known about the cytoskeletal network of human primitive erythroid cells. However, a recent membrane proteomics analysis of primary human primitive erythroblasts reveals the presence of several cytoskeletal proteins, including spectrin, ankyrin, band 3, protein 4.1, and dematin, suggesting that the cytoskeleton of primitive and definitive erythroid lineages share common structural features (Ponnusamy et al., 2012).

ONTOGENY OF PRIMITIVE AND DEFINITIVE ERYTHROPOIESIS

THE HEMANGIOBLAST ORIGIN OF PRIMITIVE ERYTHROPOIESIS

The emergence of primitive erythroid cells and a vascular network in close temporal and spatial proximity within the yolk sac suggested that these lineages might arise from common "hemangioblast" precursors (Sabin, 1920). This concept has been validated in the murine embryo using clonal assays, since cells containing both endothelial and hematopoietic potential have been localized to the primitive streak of gastrulating mouse embryos (Huber et al., 2004). Consistent with the emergence from hemangioblasts precursors, primitive erythroid potential has been derived from mesodermal cells expressing the endothelial/hematopoietic markers flk1, CD31 and Tie-2 (Ema et al., 2006). Studies of mouse and human embryonic stem cells differentiated *in vitro* toward blood cell fates also have provided evidence that hemangioblast precursors arise during gastrulation prior to the onset of hematopoietic progenitor activity (Choi et al., 1998; Zambidis et al., 2005; Kennedy et al., 2007).

DEFINITIVE ERYTHROPOIESIS- TWO DEVELOPMENTAL ORIGINS

Just as colony assays have been used to define the onset of primitive erythroid potential, a temporal and spatial analysis of definitive erythroid progenitors (BFU-E) in carefully staged mouse embryos reveals the onset of definitive erythroid potential within the yolk sac at E8.25, just prior to the onset of circulation (Palis et al., 1999). The emergence of BFU-E in the yolk sac is associated spatially and temporally with the emergence of multipotential hematopoietic progenitor cells (Palis et al., 2001), that have been termed "erythro-myeloid progenitors" (EMP). Definitive erythroid and multipotential hematopoietic progenitor activity has been generated *in vitro* from the culture of hemangioblasts, suggesting that EMP potential arises from hemangioblast precursors (Lacaud et al., 2002). Other evidence suggests that definitive hematopoietic potential emerges in the yolk sac from hemogenic endothelial cells composing part of the newly established vasculature (Li et al., 2005). More research is needed to clarify the developmental origins of definitive hematopoiesis in the mammalian embryo.

Following their initial emergence, BFU-E numbers expand rapidly in the yolk sac, are subsequently found in the bloodstream, and then in the early fetal liver (Palis et al., 1999). These yolk sac-derived definitive erythroid progenitors, when cultured *in vitro*, express predominantly adult β1- and β2-globins, but unlike their adult counterparts, they also express small amounts of the embryonic βH1-globin gene (McGrath et al., 2011). The first definitive RBCs are detected in the fetal bloodstream between E11.5 and E12.5 (McGrath et al., 2011). Mouse embryos lacking a heart beat and a functional circulation, contain similar numbers of BFU-E in the yolk sac but fail to distribute those BFU-E to the embryo proper, supporting the concept that the first definitive erythroid potential found in the fetal liver originates in the yolk sac (Lux et al., 2008). Interestingly, BFU-E in the human embryo are first detected in the yolk sac as early as 4–5 weeks gestation (Migliaccio et al., 1986). Similar to findings in the mouse, BFU-E are subsequently found in the fetal liver of the human embryo as soon as it begins to form as an organ (Migliaccio et al., 1986). These data indicate that definitive erythropoiesis first emerges after the onset of gastrulation in the yolk sac and seeds the early fetal liver.

In the adult, all blood cell lineages are derived from hematopoietic stem cells that are assayed by their ability to repopulate the entire blood system of lethally irradiated adult hosts. Using this functional assay, the first hematopoietic stem cells in the mouse embryo have been found to emerge, not from the yolk sac at E8.25, but from major arterial vessels, including the aorta, beginning at E10.5 (Kumaravelu et al., 2002). Hematopoietic stem cell activity subsequently expands in the fetal liver, peaking there at E16.5, and subsequently transitions to its long-term resident location in the bone marrow (Ema and Nakauchi, 2000; Wolber et al., 2002). This transition begins by E17.5 and 10–11 weeks in mouse and human embryos, respectively, but is not completed until after birth (Kelemen et al., 1979; Charbord et al., 1996; Blazsek et al., 2000).

Taken together, these studies support the conclusion that definitive erythropoiesis has two developmental origins. The first originates in the yolk sac of mouse and human embryos as a transient lineage that generates the first circulating definitive RBCs that emerge from the fetal liver. This transient definitive erythroid system is ultimately replaced by a second, permanent blood system, derived from hematopoietic stem cells, that provides for lifelong RBC production. However, further research is required to establish the timing of this transition from yolk sac-derived to hematopoietic stem cell-derived definitive erythropoiesis.

TRANSCRIPTIONAL REGULATION OF PRIMITIVE AND DEFINITIVE ERYTHROPOIESIS

Transcription factors play critical roles driving lineage-specific cellular maturation. The Gata1 transcription factor plays a central role in the regulation of erythroid-specific genes both in primitive and in definitive erythroid cells. Disruption of Gata1 leads to the maturational arrest of both primitive and definitive erythroid lineages at the proerythroblast stage (Pevny et al., 1995;

Fujiwara et al., 1996). Different functional domains of GATA1 are required to activate target genes in primitive vs. definitive erythroid cells (Shimizu et al., 2001), suggesting that different Gata1-containing transcriptional complexes may function in these lineages. Transcriptional regulators that can complex with Gata1 include Ldb1, FOG1, Scl/Tal1, and Lmo2. Targeted disruption of each of these genes leads to marked defects in primitive and definitive erythroid cells (Warren et al., 1994; Robb et al., 1995; Shivdasani et al., 1995; Tsang et al., 1998; Li et al., 2010).

Another important transcriptional regulator of erythropoiesis is the erythroid-specific Kruppel-like factor Klf1/EKLF (Miller and Bieker, 1993). Klf1 regulates the expression of several erythroid-specific genes including the adult and embryonic globins, alpha-hemoglobin stabilizing protein, heme biosynthetic enzymes, several transcription factors, as well as cytoskeletal proteins and blood group antigens that are expressed both in primitive and in definitive erythroid cells (Hodge et al., 2006; Nilson et al., 2006; Basu et al., 2007). Loss of even one allele of Klf1 significantly decreases the expression of Ter119 on the surface of primitive erythroblasts (Isern et al., 2010). Consistent with Klf1 regulation of multiple cytoskeletal genes, Klf1-null primitive erythroblasts display markedly abnormal cell membranes and ruffled cell surfaces.

While the primitive and definitive erythroid lineages share many transcription factors central to erythropoiesis, there are also several transcriptional regulators that are differentially expressed and function differentially in embryonic vs. fetal/adult erythroid lineages. Targeted disruption of the Myb gene completely blocks the maturation of definitive erythroblasts in the fetal liver but has no discernable effect in primitive erythropoiesis (Mucenski et al., 1991; Tober et al., 2008). Interestingly, Myb-null mouse embryos die of progressive anemia, but only after E15.5, indicating that the primitive erythroid lineage can support survival of the mouse fetus throughout most of its gestation. Humans with Trisomy 13 have persistence of embryonic and fetal hemoglobins associated with dysregulation of the Myb gene and upstream microRNas in fetal erythroblasts (Sankaran et al., 2011). Sox6 and Bcl11A are additional differentially expressed transcriptional regulators that down-regulate embryonic and fetal globin gene expression (Yi et al., 2006; Xu et al., 2010). Other transcription factors differentially expressed between primitive and definitive erythroid lineages have been identified through comparative bioinformatic analyses (Kingsley et al., 2013), however, functional studies confirming their differential expression and potential functions in the regulation of erythropoiesis have not been reported.

The complex interaction of transcription factors in the regulation of erythroid development has been modeled (Swiers et al., 2006). A summary of some of the key transcription factors regulating primitive and definitive erythropoiesis is provided in **Table 1**. In addition to the action of master erythroid transcription factors, it has recently been recognized that interferon regulatory factors, including IRF2 and IRF8, cooperate with Gata1 and Tal1, to regulate adult, but not fetal, human erythropoiesis (Xu et al., 2012). Interestingly, Irf8 is not expressed in primitive erythroblasts and IFNγ inhibits adult mouse definitive, but not primitive, erythroid colony formation (Greenfest-Allen et al., 2013). These data, taken together, suggest that inflammatory signals regulate adult, but not embryonic or fetal, erythropoiesis.

Several global gene expression analyses derived from primary primitive or definitive erythroid cells at various stages of maturation are available (Miller, 2004; Redmond et al., 2006; Isern et al., 2011; Merryweather-Clarke et al., 2011). Of note, a comparative analysis of global gene expression in similarly staged primitive, fetal definitive, and adult definitive erythroid cells has recently been published (Kingsley et al., 2013). A user-friendly website (http://www.cbil.upenn.edu/ErythronDB) has also been established, making these data readily available to the scientific community to facilitate comparative expression studies. The differential expression of globin genes, as well as several of their transcriptional regulators, have served as the primary genetic feature distinguishing primitive and definitive erythropoiesis (**Table 1**). In addition, several aquaporin gene family members were recently identified to be differentially expressed in primitive vs. adult definitive mouse erythroblasts (Kingsley et al., 2013). The expression of aquaporins 1 and 9 were confirmed in adult RBCs, while the specific expression of aquaporins 3 and 8 in primitive, but not adult definitive, erythroid cells correlates with their ability to accumulate reactive oxygen species when exposed to exogenous hydrogen peroxide. These studies raise the possibility that primitive erythroid cells may serve as a sink to protect the early embryo from free radical injury. Comparative global gene expression studies should continue to provide new insights into the biology of primitive and definitive erythroid maturation.

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Of macrophages and red blood cells; a complex love story

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Macrophages tightly control the production and clearance of red blood cells (RBC). During steady state hematopoiesis, approximately 10^{10} RBC are produced per hour within erythroblastic islands in humans. In these erythroblastic islands, resident bone marrow macrophages provide erythroblasts with interactions that are essential for erythroid development. New evidence suggests that not only under homeostasis but also under stress conditions, macrophages play an important role in promoting erythropoiesis. Once RBC have matured, these cells remain in circulation for about 120 days. At the end of their life span, RBC are cleared by macrophages residing in the spleen and the liver. Current theories about the removal of senescent RBC and the essential role of macrophages will be discussed as well as the role of macrophages in facilitating the removal of damaged cellular content from the RBC. In this review we will provide an overview on the role of macrophages in the regulation of RBC production, maintenance and clearance. In addition, we will discuss the interactions between these two cell types during transfer of immune complexes and pathogens from RBC to macrophages.

Keywords: red blood cell, macrophages, spleen, erythropoiesis, phagocytosis

FROM ERYTHROBLASTIC ISLANDS TO CLEARANCE

During their development and mature life, red blood cells (RBC) interact numerous times with macrophages, first during their development in the bone marrow, later in the blood stream with macrophages in the liver and spleen. All of these interactions are essential for efficient production under different conditions, to maintain RBC homeostasis or to ensure the correct removal of aged or damaged RBC. In this review, an overview of the different processes in which RBC-macrophage interactions play an important role is given.

THE ERYTHROBLASTIC ISLAND; A LYING-INROOM FOR EFFECTIVE ERYTHROPOIESIS

Adult erythropoiesis is a tightly regulated process which occurs in the bone marrow. It consists of several developmental stages: hematopoietic stem cell, burst- forming unit- erythroid (BFU-E), colony- forming unit- erythroid (CFU-E), proerythroblast, basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, reticulocyte and ultimately to mature RBC (Manwani and Bieker, 2008; An and Mohandas, 2011). Erythrocyte production is regulated by a negative feedback loop where oxygen levels determine plasma levels of erythropoietin (Epo). Even though there are a number of growth factors known to participate in the regulation of erythropoiesis, Epo has been identified as the master regulator of RBC production (Ji et al., 2011). Epo drives RBC precursor proliferation and differentiation and can prevent erythroblast apoptosis (Koury and Bondurant, 1990). Furthermore, terminal erythropoiesis has been reported to take place in a specialized microenvironment called the erythroblastic island. Erythroblastic islands were first described in 1958 by Besis who characterized them by analysing transmission electron micrographs of bone marrow sections. He showed a structure containing a macrophage surrounded by developing erythroblasts (Bessis, 1958) and concluded that macrophages actively participate in erythroid development by providing iron for heme synthesis and by phagocytosing expelled nuclei during final erythroid differentiation (Bessis and Breton-Gorius, 1962). Moreover, in 1972 a functional role of erythroblastic islands was demonstrated by comparing erythroblastic islands of normal rats and hypertransfused rats and showing that hypertransfused rats exhibit a significant reduction in erythroblast islands numbers by using 3D electron microscopy (Mohandas and Prenant, 1978). This finding suggests that suppression of erythropoiesis by means of RBC transfusion would result in diminished erythroid island formation, linking erythropoiesis rate to the number of erythroblastic islands. Erythroblastic islands have been described during primitive erythropoiesis as well. Even though erythroblasts in the yolk sac do not require a specialized microenvironment for development, they attach closely to structures highly similar to erythroblastic islands (McGrath et al., 2008). Moreover, erythroblastic islands have been reported in other sites for definitive erythropoiesis such as fetal liver and splenic red pulp (Manwani and Bieker, 2008).

Structurally, unlike megakaryocytes which are situated close to bone marrow sinusoids, to ensure fast egress into circulation when platelets are needed, erythroblastic islands are unevenly distributed inside the marrow, with islands adjacent or distant from the sinusoids. However, it should be noted that *in vivo* studies on erythroblastic islands in humans is virtually impossible, therefore a lot of experiments have been performed using animal models. In a study dissecting rat bone marrow, quantitative light and electron microscopy analysis shows that nonadjacent islands accommodate more pro-erythroblasts, while on the other hand islands situated next to sinusoids contain more differentiated

erythroblasts (Yokoyama et al., 2003). This interesting observation proposes that erythroblastic islands are capable of migrating towards bone marrow sinusoids as erythroid precursors mature. It is possible that interactions between erythroblast and central macrophage trigger a cascade leading to the release of macrophage proteases, which would aid extracellular matrix remodeling, and hence island progression to the sinusoid. Moreover, erythroblasts can potentially attach and detach from one central macrophage to another, thus facilitating their movement further to sinusoids. Nevertheless, the interaction between macrophage and differentiating erythroid precursors appear to be essential throughout erythropoiesis.

ROLE OF MACROPHAGES IN ERYTHROPOIESIS

Despite the fact that erythroblastic islands were described a few decades ago, understanding of the interactions between macrophages and erythroblasts during erythropoiesis is incomplete. To begin with, the specific erythroblast island cellular composition can vary depending on the species. Evidence obtained from tissue sections of rat femur shows roughly 10 erythroblasts per island (Yokoyama et al., 2002), while islands collected from human bone marrow can contain 5–30 erythroblasts surrounding a central macrophage (Lee et al., 1988).

As mentioned earlier, macrophages were proposed to promote erythropoiesis by directly transferring iron to erythroid progenitors (Bessis and Breton-Gorius, 1962). It should be noted that splenic red pulp macrophages are mainly responsible for iron return to bone marrow from recycling of senescent and damaged erythrocytes, after catabolism of hemoglobin molecules. Recently it was demonstrated in an erythroblastic island culture that ferritin produced by macrophages is released by exocytosis and engulfed by erythroblasts via endocytosis (**Figure 1**). Once inside the erythroblast, iron is released from ferritin upon acidification and proteolysis, thus being subsequently available for heme production in the erythroid precursor cell (Dautry-Varsat et al., 1983; Leimberg et al., 2008; Hentze et al., 2010; Li et al., 2010b).

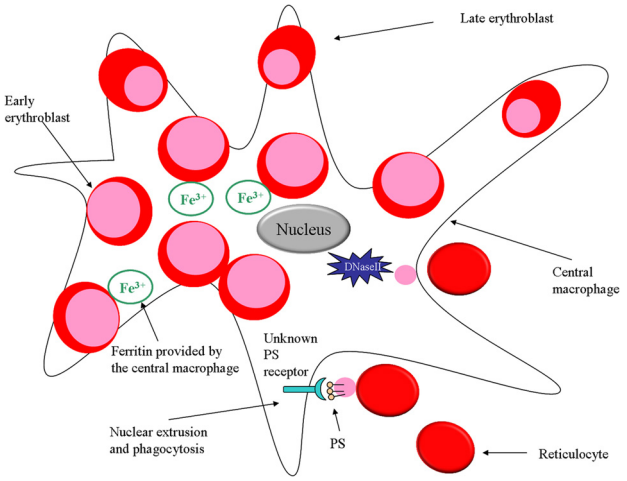

FIGURE 1 | Role of macrophages in erythropoiesis. In the erythroid niche, macrophages not only provide iron for developing erythroblasts but also phagocytose expelled nuclei. Furthermore, the macrophage protein DNaseII is important for breakdown of nuclei that are expelled by erythroblasts.

There are several examples in literature showing that macrophages not only promote erythropoiesis by providing iron, but also by directly stimulating proliferation and survival of erythroblasts. When erythroblasts are cultured *in vitro*, the erythroid precursors attached to macrophages are subjected to enhanced proliferation compared to non-attached erythroblasts proposing that macrophages may augment the response to Epo upon direct interaction with erythroblasts (Rhodes et al., 2008).

It has been demonstrated that abnormal macrophage differentiation can have a direct effect on erythroblastic island function. For instance, when retinoblastoma tumor suppressor (Rb) protein is knocked out in a mouse model, fetuses die *in utero* due to anemia (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Rb is a nuclear factor that regulates cell cycle transition from G1 to S phase and is critical for macrophage differentiation (Iavarone et al., 2004).

Cytoskeletal-associated protein palladin has also been implicated in macrophage function. It is a protein that localizes in focal adhesions of stress fibers together with α- actinin, thus promoting cytoskeletal dynamic rearrangements and adherence to the extracellular matrix. Knocking out palladin in a mouse model is embryonic lethal due to anemia caused by erythroblast cell death and aberrant terminal erythroid differentiation. Fetal liver erythroblastic island integrity is compromised and *in vitro* erythroblastic island formation is perturbed in palladin$^{-/-}$ mice due to an intrinsic macrophage defect (Liu et al., 2007).

In addition, the macrophage transcription factor c-Maf has been identified as a critical component in definitive erythropoiesis in fetal liver. Deletion of c-Maf leads to severe erythropenia *in utero* and significant reduction in fetal liver erythroblastic island formation compared to wild type. The observed defective erythropoiesis seems to be due to an abnormal erythroid niche and not to a cell autonomous effect (Kusakabe et al., 2011). These examples clearly show that macrophages are crucial participants in erythroid development as targeted deletion of enzymes, proteins and transcription factors responsible for macrophage proliferation and survival ultimately results in perturbed erythroid niche formation and defective erythropoiesis.

DIRECT INTERACTIONS BETWEEN ERYTHROBLASTS AND MACROPHAGES WITHIN ERYTHROBLASTIC ISLANDS

Needless to say, the function and integrity of erythroblastic islands is tightly related to the molecular interactions occurring between erythroid precursors and the central macrophage. Erythroblasts express a myriad of adhesion molecules throughout their differentiation, which not only facilitate adhesion to extracellular matrix proteins such as fibronectin and laminin, but also attachment to the central macrophage. The first molecule identified on the surface of both central macrophages and erythroblasts is Erythroblast macrophage protein (Emp), a protein that promotes binding between the two cell types (Hanspal and Hanspal, 1994) (**Figure 2**). In erythroblastic island cultures absence of Emp leads to aberrant erythropoiesis and increased levels of apoptosis (Hanspal et al., 1998), suggesting that the direct association between the central macrophage and the erythroblasts is essential

for erythroid maturation and prevention of cell death. In support of those findings are the *in vivo* experiments performed with Emp deficient mice, which show that those animals die before birth owing to severe anemia (Soni et al., 2006). Next, another important molecular interaction found in erythroblastic islands occurs between the α4β1 integrin (Very Late Antigen 4; VLA-4) on erythroblasts and vascular cell adhesion molecule 1 (VCAM-1) on central macrophages (**Figure 2**). The biological significance of this receptor pair is underlined in experiments in which erythroid island formation is perturbed by antibodies against α4β1 integrin or VCAM-1 (Sadahira et al., 1995). Studies in mice have shown that integrins have a pivotal role in stress erythropoiesis (Ulyanova et al., 2011). In addition, intercellular adhesion molecule 4 (ICAM-4) expressed on erythroblasts and αV integrin present on macrophages (**Figure 2**) have a vital function in maintaining island integrity, since disrupting the binding between the two molecules using synthetic peptides leads to a diminished number of erythroblastic islands. In an *in vivo* model, utilizing ICAM-4 deficient mice, reduced island formation is observed as well (Lee et al., 2006). Moreover, a secreted form of ICAM-4 possibly regulates terminal erythropoiesis by competing with surface ICAM-4 for αV integrin on central macrophages preventing the interaction between erythroblasts and macrophage (Lee et al., 2003).

CD163 is another macrophage receptor that interacts with erythroblasts (Fabriek et al., 2007) (**Figure 2**). CD163 is well-known to scavenge hemoglobin-haptoglobin complexes, thus clearing free hemoglobin from circulation (Kristiansen et al., 2001). CD163 contains an erythroblast adhesion motif as well, which mediates binding of macrophages to erythroid precursors facilitating erythroblast expansion and survival (Fabriek et al., 2007). Future studies are required to further characterize the contribution of the direct interactions between erythroblasts and macrophages on island structural integrity and on signaling pathways during erythropoiesis. For instance, the specific receptor/ligand pairs on erythroblasts and macrophage that are involved in cell-cycle regulation during erythropoiesis have not yet been identified. Likely candidates are macrophage membrane protein Ephrin-2 (HTK ligand) binding erythroid receptor EphB4 (HTK) (**Figure 2**) (Inada et al., 1997; Suenobu et al., 2002) and c-kit ligand interacting with c-kit on erythroblasts (Muta et al., 1995). Both macrophage surface molecules have been shown to augment erythroid proliferation.

SOLUBLE FACTORS IMPORTANT IN ERYTHROBLASTIC ISLANDS

Interestingly, there is experimental evidence suggesting that erythroblasts might modulate island integrity by secreting angiogenic factors such as vascular endothelial growth factor A (VEGF-A) and placental growth factor (PGF) (Tordjman et al., 2001) (**Figure 3**). One can hypothesize that release of these molecules can contribute to the reticulocytes' egression into the vasculature by regulating the stability of endothelial junctions. Moreover, even though erythroblasts do not express receptors for VEGF-A and PGF on their surface, central macrophages do, suggesting that erythroblasts may secrete those factors as paracrine modulators of macrophage proliferation and survival in the erythroid niche.

On the other hand, growth arrest-specific 6 (Gas-6) released by erythroblasts has been proposed to modulate the erythroid microenvironment during erythropoiesis. Gas-6 is a molecule normally associated with proliferation and survival of non-erythroid cells. However, erythroblasts can secrete Gas-6 in response to Epo, thus positively regulating Epo signaling through

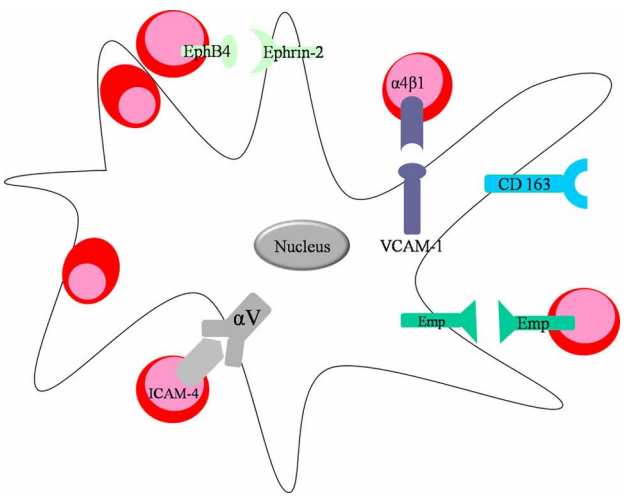

FIGURE 2 | Direct interactions between the central macrophage and developing erythroblasts. Macrophages express VCAM-1 and integrin αV on their surface facilitating interaction with erythroblasts through integrin α4β1 and ICAM-4, respectively. Moreover, both erythroid cells and macrophages express Emp on their surface promoting their interactions in the niche. Central macrophage and erythroblast can also interact via the ligand-receptor pair EphB4 (expressed on the surface of erythroblasts) and Ephrin-2, found on macrophages.

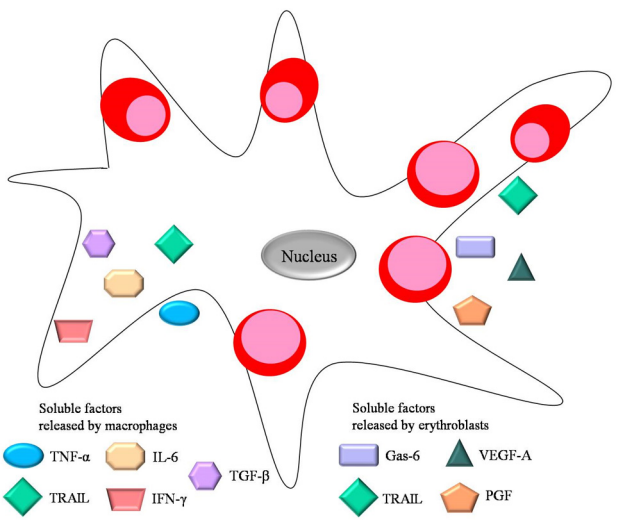

FIGURE 3 | Soluble factors important for erythropoiesis. Both central macrophage and erythroblast secrete soluble factors during erythropiesis. These include the negative regulators TNF-α, TGF-β, IL-6, IFN-γ released by the central macrophage, and the positive regulators Gas-6, VEGF-A and PGF released by erythroblasts. TRAIL is a negative regulator secreted by both erythroid cells and macrophages.

phosphoinositide 3 kinase (PI3K) and Akt activation (Angelillo-Scherrer et al., 2008).

Furthermore, there are several soluble factors secreted by macrophages within the erythroblastic island that negatively regulate erythropoiesis. These include cytokines, chemokines and interleukins including interleukin 6 (IL-6), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ), all of which are associated with chronic inflammation and tumor progression. For instance, patients suffering from chronic inflammation have high levels of inflammatory cytokines in the bone marrow which inhibit erythropoiesis (Means, 2004). The mechanism by which TNF-α released by macrophages suppresses erythropoiesis involves caspase-mediated cleavage of GATA-1, a pivotal transcriptional regulator of erythroid development. This leads to apoptosis (De Maria et al., 1999) or delayed proliferation (Dai et al., 2003) of the erythroid progenitors. During terminal differentiation GATA-1 is normally protected from caspase cleavage by heat shock protein 70 (Hsp-70) (Ribeil et al., 2007). Thus Hsp-70 can regulate erythropoiesis by preventing the induction of apoptosis which would negatively influence RBC production. Furthermore, secreted TNF-α can trigger release of metalloproteases by macrophages in order to remodel extracellular matrix in other tissues, however a similar event occurring in the context of erythroblastic islands would have deleterious effects on the microenvironment's integrity.

Bone marrow macrophages isolated from myelodysplastic syndrome (MDS) patients release higher levels of TNF-α compared to macrophages from healthy donors (Flores-Figueroa et al., 2002). Moreover, they present with an increased apoptotic index, suggesting an abnormal macrophage function inside the erythroid niche in MDS.

Additionally, TGF-β released by macrophages is known to block erythroblast proliferation and survival via a mechanism different than apoptosis, and at the same time enhances erythroid differentiation (Zermati et al., 2000). TGF-β can activate Rho and Rac GTPases, influencing cell cytoskeletal stability and organization in various cell types (Maddala et al., 2003), including erythroblasts, which require stable cytoskeletal integrity for normal development. Increased levels of the inflammatory cytokine IFN-γ can lead to secretion of TNF-related apoptosis inducing ligand (TRAIL), by both macrophages and erythroblasts (Zamai et al., 2000). TRAIL blocks erythroid differentiation by a activating the ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) pathway (Secchiero et al., 2004). All soluble factors discussed above are depicted in **Figure 3**. Notably, many studies on erythroblastic islands and erythroid proliferation and survival manage to establish the crucial link between macrophage and erythroblast interactions and their effects on erythroid niche development.

MACROPHAGES PHAGOCYTOSE NUCLEI EXPELLED FROM ERYTHROBLASTS

During the final stage of terminal erythroid differentiation, the erythroblast expels its nucleus as part of its maturation into a reticulocyte. The macrophage has a critical role during this process since it will phagocytose the expelled nucleus, aiding erythropoiesis (Seki and Shirasawa, 1965; Skutelsky and Danon, 1972). Both the macrophage and the erythroblast/reticulocyte are equipped with adhesion molecules promoting the retention of the nucleus on the surface of the macrophage before phagocytosis takes place. It has been shown that Emp (Soni et al., 2006) and β1 integrin (Lee et al., 2004) predominantly distribute on the nucleus after expulsion, thus maintaining the interaction between the nucleus and the macrophage. Moreover, studies performed with fetal liver erythroblasts demonstrate that expelled nuclei expose phosphatidylserine (PS) on their surface for 10 min after expulsion (Yoshida et al., 2005) (**Figure 1**). This observation corroborates with the finding that the time frame between nucleus expulsion and phagocytosis is 10 min (Allen and Testa, 1991), which suggests that PS might be assisting in the adhesion of the nucleus to the macrophage prior to phagocytosis. PS is a membrane component normally situated on the inner leaflet of the cell membrane. An ATP-dependent aminophospholipid translocase enzyme maintains the lipid asymmetry by keeping PS on the inside of the plasma membrane. PS exposure on the cell surface is considered an apoptotic signal targeting cells undergoing cell death for clearance by phagocytes expressing PS receptors. Moreover, PS externalization can be a direct effect of ATP depletion in the cell. In addition, Yoshida and colleagues have demonstrated that expelled nuclei expose PS and lack ATP (Yoshida et al., 2005). Furthermore, the PS-binding protein lactadherin (also known as MFGE8) which normally serves as a bridging molecule between an apoptotic cell and a phagocyte (Hanayama et al., 2002), has been shown to be crucial for phagocytosis of extruded erythroblast nucleus as well. A mutated form of lactadherin, not being able to bind PS, inhibits phagocytosis of expelled nuclei (Yoshida et al., 2005).

Another study that suggests an important role for macrophages for the phagocytosis and degradation of expelled nuclei during erythropoiesis made use of DNase II deficient mice. DNase II is an enzyme necessary to degrade nuclear DNA after phagocytosis and proves to be essential for erythropoiesis (Kawane et al., 2001) (**Figure 1**). It was shown that fetal liver macrophages from DNase II deficient mice are unable to degrade the ingested nuclei and that DNase II-null mice die *in utero* due to severe anemia.

MACROPHAGES IN ERYTHROPOIESIS: IN HEALTH AND DISEASE

Despite the fact that our knowledge of macrophage-erythroblast interactions in erythroblastic islands and their role in erythroid development is expanding, it is important to realize that many of the experiments have been conducted *in vitro*. In a recent paper Chow and colleagues elegantly show that $CD169^+$ macrophages promote erythropoiesis in steady state and under stress *in vivo* (Chow et al., 2013). CD169 was first described to be a marker of central macrophages in the erythroblastic island more than two decades ago (Crocker et al., 1990). In the study by Chow et al. depletion of $CD169^+$ macrophages leads to a decreased number of erythroblasts in the bone marrow and mild iron-deficiency anemia. Furthermore, $CD169^+$ macrophages appear to be essential for recovery from hemolytic anemia, acute blood loss and myeloablation. On the other hand, macrophage depletion can rescue the phenotype of polycythemia vera in a $JAK2^{V617F}$- driven mouse model. These findings suggest that macrophages are not

only a critical component during erythroid maturation in steady state, but also during stress and disease.

To support the speculation that macrophages might also have a function in erythropoiesis in the context of disease and to further characterize their importance in erythropoiesis *in vivo*, Ramos and colleagues show that macrophages regulate erythroid development in polycythemia vera, β-thalassemia and anemia (Ramos et al., 2013). Chemical depletion of macrophages by clodronate liposome administration prevents mice from recovering from induced anemia, suggesting an essential function of macrophages in promoting stress erythropoiesis *in vivo*. Conversely, macrophage depletion not only improves the phenotype of polycythemia vera and reverses the pathological aspects of the disease, but also alleviates anemia caused by β-thalassemia. These results propose an important dual role of macrophages in physiological and pathological erythropoiesis *in vivo*. Both studies suggest that macrophages exert two seemingly contradictory actions on erythropoiesis. On one hand, macrophages are indispensable for stress erythropoiesis *in vivo*. In their absence erythroid production in the bone marrow and spleen in response to bleeding is impaired. However, macrophages can also be deleterious in the context of polycythemia vera and β-thalassemia, since depletion of macrophages leads to a decreased disease pathology. Moreover, *ex vivo* cultured human macrophages from polycythemia vera patients promote proliferation of human erythroblasts and diminish differentiation. This suggests a function for macrophages in disease progression since polycythemia vera is characterized by an overactive erythron and excessive erythropoiesis (Ramos et al., 2013). These findings might pave the way to future therapies implementing macrophage depletion in the treatment of erythroid disorders like polycythemia vera and β-thalassemia. These and other studies demonstrate the importance of erythroblastic islands and more specifically, the interaction between macrophage and erythroblasts for RBC maturation in physiological and pathological conditions. Future experiments are necessary to examine in more detail the involvement of macrophages in red blood cell production in steady state and disease. Even though, animal models are necessary to illustrate the *in vivo* situation, one should take these studies into consideration with caution. It should be noted that erythroid development within erythroblastic islands differs between mice and men.

INTERACTION OF RBC AND MACROPHAGES IN THE BLOODSTREAM

After they are produced in the bone marrow, RBC remain in circulation for roughly 120 days. Throughout their life span RBC pass the liver and the spleen numerous times where they encounter resident macrophages (Crosby, 1959). The interactions between macrophages and RBC taking place in liver and spleen are important for RBC homeostasis and ultimately for the removal and degradation of aged RBC at the end of their life span (Mebius and Kraal, 2005). In addition, macrophages take up immune complexes and pathogens bound to complement receptor 1 (CR1) on the RBC and can clear intracellular pathogens such as *Plasmodium* from the RBC, leaving the RBC intact and allowing the return of the RBC into circulation (Wilson et al., 1987). The different molecular interactions that are important for these different processes are discussed below.

THE REMOVAL OF INTRACELLULAR INCLUSIONS BY MACROPHAGES OF THE SPLEEN

The macrophages of the spleen have a remarkable function that enables them to remove unwanted damage from the RBC membrane, leaving the RBC intact (Crosby, 1957; Schnitzer et al., 1972). Removal of these intracellular inclusions seems to occur within the open circulation where the RBC are also checked for their loss of deformability to check for age. To achieve this, RBC must pass through the endothelial slits of the sinus to reenter the blood circulation. During this course, cells that are non-deformable will be removed from the circulation by residential macrophages. In the mean-time all inclusion bodies are also being removed. In splenectomized patients or in patients with a non-functional spleen, phagocytosis of the inclusion bodies fails and results in a retention of a variety of intracellular inclusions within the RBC, such as Howell-jolly bodies (inclusions of nuclear chromatin remnants) (Wilkins and Wright, 2000), Heinz bodies (inclusions of denatured hemoglobin caused by oxidative damage) (Wilkins and Wright, 2000) siderocytes (RBC containing granules of iron that are not part of the cell's hemoglobin) (Wilkins and Wright, 2000) and Pappenheimer bodies inclusion bodies formed by phagosomes that have been engulfing excessive amounts of iron (Wilkins and Wright, 2000).

Back in 1957 Crosby already showed that when siderocytes, tagged with radioactive chromium, were injected into a healthy patient with a functional spleen, there was a decline in siderocyte count without the loss of chromium labeled RBC. When the same amount of siderocytes was injected into a splenectomized patient, the amount of siderocytes remained unaltered during the 24-h period of observation. This study hereby demonstrated that passage through the spleen can lead to clearance of damage that is accumulating in the circulating RBC. Furthermore, it revealed that processing of damage from RBC can take place while leaving the RBC intact. Thus, it seems that the spleen and the residential macrophages are highly important in maintaining RBC "healthy." RBC are of course unable to synthesize new proteins, and although equipped with enzyme systems to counteract the potential toxic effects of the oxygen they transport, they will sustain oxidative damage throughout their life resulting in the formation of Heinz bodies (Harley, 1965). The molecular mechanism that underlies the removal of inclusion bodies is largely unknown. In Willekens et al. (2003) presented an analogy to the removal of Heinz bodies when discussing RBC that lose hemoglobin through vesiculation. Via the process of RBC vesiculation the RBC loses aggregated hemoglobin, which is important to maintain the homeostasis of RBC, increases in density and becomes smaller (Piomelli and Seaman, 1993). It was suggested that this process is also facilitated by the macrophages of the spleen, in which older cells vesiculate more than younger ones. Clearly, macrophages play a pivotal role in the clearance of damaged content from circulating RBC (Crosby, 1957; Willekens et al., 2003) and vesiculation is an interesting and plausible mechanism to explain the efficient removal of damaged content while leaving the RBC intact (Wilson et al., 1987). The molecular mechanism by which macrophages in the spleen would be facilitating RBC vesiculation is still unknown. Ultrastructural studies of spleens from monkeys infected with *Plasmodium knowlesi* suggest that

the spleen also removes malaria parasites from red cells, in which once again phagocytes play the main role (Schnitzer et al., 1972). In addition, interesting work by Buffet and colleagues has pointed out that the removal of malaria parasites from RBC occurs in the red pulp of the spleen, using perfused human spleens to proof this point (Buffet et al., 2006). Several studies suggest that due to this function the spleen plays a major protective role in naïve patients (Bachmann et al., 2009; Munasinghe et al., 2009). They all showed that disease severity, parasitemia and mortality were higher in splenectomized patients. This supports the hypothesis that without a functional spleen, there will be no splenic retention which could explain why the outcome in splenectomized patients compared to patients who have a functional spleen is worse.

RBC REMOVAL BY MACROPHAGES

Residential macrophages of the spleen are able to scrutinize passing RBC and remove those from the circulation that are at the end of their lifespan or have sustained damage beyond repair (Mebius and Kraal, 2005). For example: deformed RBC that have been produced by mistake by the bone marrow or RBC that are affected by hereditary spherocytosis will be taken up by spleen macrophages in the red pulp (Crary and Buchanan, 2009).

At present there is no consensus as to how red pulp macrophages determine which RBC need to be cleared and which can be repaired and/or maintained. It is difficult to identify RBC that are destined to be cleared *in vivo* due to the fact that RBC that are carrying a removal signal will most likely be phagocytosed and hence will no longer be available for analysis. In addition, the proportion of RBC that is daily cleared is a mere 0.8% per day, thus leaving only a very small number of RBC that will carry the removal signals at any given moment a blood sample is taken. Therefore, most of the theories on possible removal signals for RBC phagocytosis are based on *in vitro* work or on data generated in animal models.

The RBC aging phenotype, according to our current knowledge, is associated with a decline in metabolic activity and progressive membrane remodeling, due to for example oxidative stress and vesiculation. Concomitantly, the RBC becomes smaller and more dense (Piomelli and Seaman, 1993). Despite these cumulative events, the removal signals do not seem to gradually accumulate on RBC. On the opposite, they appear as a snap, rapid and non-linear cascade of events at the terminal stage of the aging process, probably shortly before RBC are removed by macrophages (Franco, 2009). Taking into account that RBC are unable to synthesize new proteins, all "removal" markers must derive from modifications in pre-existing molecules or to the acquisition of plasma-derived opsonins directed against these modifications. Although RBC do not undergo classical apoptosis since they do not contain a nucleus, mitochondria or other cellular organelles, the process they undergo has already been termed "eryptosis" since it exhibits many similarities with programmed cell death (Lang et al., 2005). For instance, it is highly likely that phagocytosis of senescent RBC will be non-inflammatory. Thus far, there have been several mechanisms postulated for the clearance of senescent RBC by macrophages.

BAND 3-BASED CLEARANCE MECHANISM

Band 3, a transmembrane protein that constitutes 25% of the total amount of RBC membrane proteins, has been postulated to be the major target of natural occurring antibodies (NAbs) of the IgG isotype and might be a central step in clearance of senescent and damaged RBC that is mediated by macrophages (Lutz, 2004; Arese et al., 2005; Kay, 2005) Band 3 has two different domains: the membrane spanning domain that catalyses anion exchange and is recognized by Nabs (after clustering) (**Figure 4**), and a cytoplasmic domain that regulates the structure and function of the RBC by binding different proteins (Zhang et al., 2000; Pantaleo et al., 2008). There is still debate about the mechanism that leads to formation of the epitope on band 3 that results in binding of NAbs. It is thought that oxidative damage to hemoglobin, occurring throughout its lifecycle, and the following formation of hemichromes which bind to band 3, can in time lead to band 3 clustering (Pantaleo et al., 2008; Arashiki et al., 2013). NAbs show an enhanced affinity to band 3 clusters (Low, 1986; Mannu et al., 1995; Hornig and Lutz, 2000). Another hypothesis is that proteolytic degradation of band 3 is essential to shape the band 3 epitopes recognized by NAbs (Kay, 2004).

However, NAbs are not efficient opsonins, due to their low affinity and low circulation numbers. It has been hypothesized that phagocytosis of RBC can be enhanced by the activation of the classical pathway of the complement system after NAb binding. These NAbs preferentially generate $C3b_2$–IgG complexes in the presence of active complement (Lutz et al., 1993). Once the

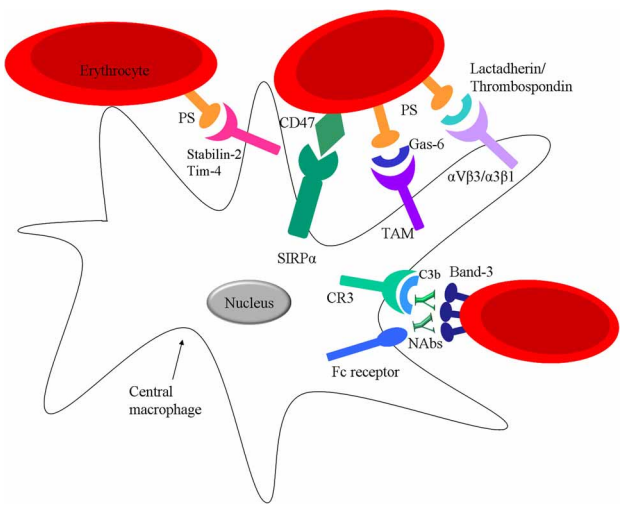

FIGURE 4 | Interactions between mature RBC and spleen macrophages. RBC can interact with spleen macrophages via direct receptor ligand pairing or via bridging molecules. Ageing RBC express PS on their surface which can directly bind to Stabilin-2 or Tim-4 on the macrophage or via opsonins such as Gas-6, lactadherin or thrombospondin-1. RBC express CR1 on their surface which can bind C3b osponized particles and further facilitate interaction with spleen macrophages via CR1 and CR3. Nabs can bind Band-3 on the surface of RBC targeting the cell for clearance via Fc receptors on the spleen macrophages. Moreover, RBC express CD47 which binds SIRPα on macrophages.

classical pathway is activated a significantly lower amount of NAbs is needed for induction of phagocytosis (Lutz et al., 1990). This is because once opsonized with C3b, C3b will form complexes with NAbs that are more resistant to inactivation factors such as: H and I. Red pulp macrophages express CR1 (C3b-receptor, CD35) and CR3 (iC3b-receptor, CD11b/CD18) (Burger and van Bruggen, unpublished) that would enable them to recognize and phagocytose complement opsonized RBC (**Figure 4**). However, phagocytosis through either of these receptors usually results in the secretion of pro-inflammatory cytokines, although the opposite effects, thus an inhibition of the secretion of pro-inflammatory cytokines has also been shown (Morelli et al., 2003). The effect of complement receptor-mediated phagocytosis on cytokine secretion in macrophages of the spleen is currently under investigation.

REMOVAL OF AGED RBC BY PHOSPHATIDYLSERINE RECOGNIZING RECEPTORS ON MACROPHAGES

In healthy cells phosphatidylserine (PS), is normally found on the inner leaflet of the RBC membrane. However, when apoptosis is induced a large increase is seen in the amounts of PS exposed at the cell surface. This increase in PS expression is proposed to be an "eat me" signal for phagocyte recognition of an apoptotic cell, resulting in a non-inflammatory clearance of the dying cell (Fernandez-Boyanapalli et al., 2009).

In an *in vivo* experiment, an increase in PS exposure is seen with RBC age, correlated to the RBC clearance from the circulation (Boas et al., 1998). For a long time it has been proposed that apoptotic cells that express PS can be cleared from circulation via macrophages by recognizing them through specific PS-receptors (Li et al., 2003). Yet recently, various receptors have been identified that can mediate binding and phagocytosis of apoptotic cells by the recognition of PS on these cells such as Tim1, Tim4 and Stabilin-2 (Kobayashi et al., 2007; Park et al., 2008). In addition, there are several bridging molecules such as the plasma proteins: lactadherin, Gas-6 and protein S, that have been described to bind to PS and direct PS to receptors on phagocytes, $\alpha_v\beta_{3/5}$ integrins and receptors of the TAM receptor family and mediate clearance of PS-positive cells (Raymond et al., 2009). Of these receptors, at least Axl, Tim4 and Stabilin-2 are expressed in red pulp macrophages (Burger and van Bruggen, unpublished) (**Figure 4**). Thus, this opens the possibility that PS-exposing RBC are cleared in the spleen by phagocytosis by one or more of these PS or PS/ligand receptor pairs. As was already mentioned above, the loss of phospholipid asymmetry and the subsequent exposure of PS on the RBC surface in apoptotic cells may be a general trigger for RBC removal. It also seems that upon RBC storage the susceptibility to stress-induced PS exposure increases, thereby causing a considerable fraction of the RBC to be susceptible to removal after transfusion (Bosman et al., 2011).

Of interest, N-Acetyl-L-Cysteine (NAC) prolong the half-life of circulating mouse erythrocytes *in vivo* RBC drawn from mice that were subsequently treated with NAC exhibited a significantly higher survival rate after the intravenous injection into the sibling mice than those RBC without an NAC treatment (Ghashghaeinia et al., 2012).

CD47; A MOLECULAR SWITCH FOR RBC PHAGOCYTOSIS

The immunoreceptor signal regulatory protein alpha (SIRPα), expressed by macrophages, is well-known for its ability to inhibit phagocytosis of CD47 expressing cells (Oldenborg et al., 2000; Ishikawa-Sekigami et al., 2006). The CD47-SIRPα interaction (**Figure 4**) provides a strong negative signal for phagocytosis and can function as a marker of "self" on RBC. Thus, a low level of opsonization might suffice to trigger phagocytosis of a foreign particle that does not express CD47, whereas a "self" particle, such as an RBC, would not be phagocytosed by macrophages in the spleen due to the presence of CD47 on the RBC and the resulting inhibitory signals generated upon contact with macrophage SIRPα. Interestingly, mild hemolytic anemia is seen in Rh-null or protein 4.2-deficient human individuals (Miller et al., 1987; Bruce et al., 2002), which both have strongly reduced RBC CD47 expression levels. Thus it is tempting to speculate that the hemolytic anemia in these individuals may in part be the result of reduced CD47-SIRPα inhibitory signaling to splenic macrophages, possibly in combination with the altered morphology and rheological properties of the RBC in these syndromes.

Based on this, it is of interest to determine whether a reduced expression of CD47 during RBC senescence is also involved in facilitating the uptake of these cells by macrophages. Some studies suggest that there is evidence that point in this direction. One group has found, that a fraction of older murine RBC (>30 days old) show about 20% reduced CD47 expression as compared to a fraction of younger RBC (Fossati-Jimack et al., 2002).Also in another study, RBC storage, which is known to be associated with accelerated RBC clearance following transfusion, was shown to result in loss of CD47 (Anniss and Sparrow, 2002), although we were not able to reproduce these results for RBC stored under standard Dutch blood bank conditions (Burger and van Bruggen, unpublished).These observations, in combination with the previous findings, suggest that clearance of senescent RBC may be regulated by the net result of total signaling through macrophage pro-phagocytic and inhibitory receptors.

However, in 2012 our group has shown that CD47 does not only function as a "don't eat me" signal, but can also act as an "eat me" signal (Burger et al., 2012). In particular, a subset of old RBC present in whole blood was shown to bind and to be phagocytosed via CD47-SIRPα interactions. Furthermore, our group provided evidence that experimental aging of RBC induces a conformational change in CD47 that switches the molecule from an inhibitory into an activating one. Pre-incubation of experimentally aged RBC with human serum prior to the binding assay was required for this activation. In the same study we also demonstrated that aged RBC have the capacity to bind the CD47-binding partner thrombospondin-1 (TSP-1) and that treatment of aged RBC with a TSP-1 derived particle enabled their phagocytosis by human red pulp macrophages. Finally, CD47 on RBC that had been stored for prolonged time was shown to undergo a conformational change and bind TSP-1. These findings reveal a more complex role for CD47-SIRPα interactions in RBC clearance, with CD47 acting as a molecular switch controlling phagocytosis. In addition, we were able to determine that this CD47/SIRPα pathway leading to phagocytosis of RBC is operational in human red pulp macrophages.

RBC COMPLEMENT RECEPTOR 1: MEDIATOR IN IMMUNE-ADHERENCE CLEARANCE THROUGH MACROPHAGES

Immune adherence was first described by Nelson (1953) as the immunological reaction between RBC and complement opsonized pathogens. Humans and other higher primates are unique for immune-adherence clearance (IAC) using complement receptor 1 (CR1) on the RBC membrane, which is a critical protection in host defense against blood-borne pathogens such as bacteria. For the vast majority of lower primates, not RBC, but platelets are responsible for binding and transporting circulating complement opsonized particles.

CR1 on human RBC binds complement opsonized particles bearing C3b/C4b in the circulation (Ross and Medof, 1985; Wilson et al., 1987). The opsonized particles are subsequently transported to the spleen and liver where they are removed by residential macrophages, without phagocytosing the RBC. Another group has shown in an *in vitro* study that the transfer rate of immune complexes to monocytes is accelerated once the immune complexes are bound to RBC CR1 compared to unbound opsonized immune complexes, thereby increasing the efficiency of immune complex removal (Emlen et al., 1992). Furthermore, in a mouse transgenic model, where human CR1 is expressed on murine RBC, immune adherence was shown to enhance the resistance of the host to infection with *S. pneumoniae* (Li et al., 2010a).

Recently, interesting work by Melhorn and colleagues on the interplay between RBC and phagocytes in transfer of opsonized particles has been published. It was shown that signal transduction downstream of CR1 after particle binding results in alterations in RBC membrane deformability and in clustering of CR1 on the RBC surface, which enhances binding of the opsonized particle. But more importantly, proof was provided that CR1 ligation leads to ATP secretion, which has a direct stimulatory effect on particle uptake by phagocytes (Melhorn et al., 2013). Thus, the RBC seems to play an active role in the capture of opsonized particles as well as the subsequent transfer of these particles to phagocytes.

RBC MACROPHAGE INTERACTIONS: FUTURE DIRECTIONS

Macrophages play a pivotal role in RBC production, maintenance and clearance. Although it is clear that macrophage–RBC interactions are critical in these processes, the molecular mechanisms behind many of these interactions are still elusive. The role of macrophages in the formation of erythroblastic islands is the most extensively studied process in which RBC macrophage interactions are important. The other two biological phenomena, maintenance and clearance of RBC are less well-understood. Especially, the process in which macrophages of the spleen aid the removal of inclusions in the RBC, are obscure. In the near future efforts should be made to fully understand this process as well as RBC clearance. New techniques such as intravital microscopy might be used to study these processes in detail, thereby generating knowledge that may aid to prevent unwanted RBC destruction in diseases such as β-Thalassemia or hemolytic anemia, or reduce the loss of stored donor RBC after transfusion.

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Neocytolysis: none, one or many? A reappraisal and future perspectives

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Neocytolysis is the hypothesis formulated to explain experimental evidence of selective lysis of young red blood cells (RBCs) (neocytes) associated with decreased plasma levels of erythropoietin (EPO). In humans, it appears to take place whenever a fast RBC mass reduction is required, i.e., in astronauts during the first days of spaceflight under weightlessness, where a fast reduction in plasma volume and increase in haematocrit occur. EPO plasma levels then decline and a decrease in RBC mass takes place, apparently because of the selective lysis of the youngest, recently generated RBCs (neocytes). The same process seems to occur in people descending to sea level after acclimatization at high altitude. After descent, the polycythaemia developed at high altitude must be abrogated, and a rapid reduction in the number of circulating RBCs is obtained by a decrease in EPO synthesis and the lysis of what seem to be young RBCs. *In vivo*, neocytolysis seems to be abolished by EPO administration. More recent research has ascribed to neocytolysis the RBC destruction that occurs under such disparate pathophysiologic conditions as nephropathy, severe obstructive pulmonary disease, blood doping, and even malaria anaemia. According to the theory, EPO's central role would be not only to stimulate the production of new RBCs in conditions of anaemia, as maintained by the orthodox view, but also that of a cytoprotective factor for circulating young RBCs. Why neocytes are specifically destroyed and how is this related to decreased EPO levels has not yet been elucidated. Changes in membrane molecules of young RBCs isolated from astronauts or mountain climbers upon return to normal conditions seem to indicate a higher susceptibility of neocytes to ingestion by macrophages. By limiting the context to space missions and high altitude expeditions, this review will address unresolved and critical issues that in our opinion have not been sufficiently highlighted in previous works.

Keywords: neocytolysis, erythropoietin, red cell lifespan, red cell mass regulation, red cell senescence, microgravity, space flight, mountaineering

INTRODUCTION

The red blood cells (RBCs) of mammals are non-nucleated cells that spend in the circulation a limited amount of time, after which they are removed by the reticulo-endothelial system according to a species-specific type of kinetic. This results from the superimposition of random destruction (independent of cell age) and of a senescence process. The magnitude of the random component varies in different species and in different animals within the same species. It is very pronounced in mice and rats, less pronounced in pigs, rabbits and other mammalians, and almost absent in normal human RBCs, which are recognized as senescent and removed after 120 days of circulatory life (Clark, 1988; Landaw, 1988; Brovelli and Minetti, 2003). In various haematological disorders the destruction of poorly deformable or otherwise compromised RBCs occurs at a faster rate, with spleen as the main organ involved in the process, since splenectomy often alleviates the abnormal shortening of life-span of these cells (Landaw, 1988). On the other hand, it is believed that the spleen has only a modest role in the removal of normal senescent RBCs, which were shown to be phagocytosed almost exclusively in the bone marrow (Miescher, 1956; Marton, 1970; Clark, 1988; Landaw, 1988). The mass of RBCs (RBCM) circulating at each given time is the result of a dynamic balance between the destruction of old cells and the production of new ones, which derive from precursors of the erythroid lineage. The production of precursors is regulated, in its early phase, by various factors, among which are erythropoietin (EPO), that maintains cell vitality and transmits anti-apoptotic signals, and SCF (stem cell factor), a proliferation factor (Jacobs-Helber et al., 1997; De Maria et al., 1999). At later stages, primary erythroblasts proceed through their proliferation and differentiation programs, thanks to other factors, among which osteopontin appears to play a central role as a factor of proliferation and remodeling of the cytoskeleton (Kang et al., 2008). On the other hand, it is an accepted view that, under physiologic conditions, no mechanisms exist (or are not known), which are able to decrease the RBC life-span below an established, fixed value, which, in humans, amounts to the said 120 days (corresponding to the removal of approximately 1% RBCs per day).

THE NEOCYTE AND NEOCYTOLYSIS

The neologism "neocyte" was firstly adopted in the context of transfusion medicine in the late 70s, when possible improvements in the transfusion regimens for patients with haemoglobinopathies (particularly thalassemia) were under intensive study, and one approach was based on the infusion of "neocytes" (then defined as the 50% less dense circulating RBCs, which were also assumed to comprise the youngest RBCs) with higher survival rate, with the aim at reducing the frequency of transfusions, and thus iron overload, in these patients (Propper et al., 1980). More recent evidence has shown, however, that the infusion of neocytes is less advantageous than expected (Pisciotto et al., 1986), and it is nowadays not practiced on a large scale for the long-term treatment of patients (Forget and Olivieri, 2003).

The term "neocytes" has been adopted again, more recently and in an entirely different context, with the theory proposed by Alfrey and co-workers to explain the anaemic condition that affects astronauts after space flights ("space anaemia") (Alfrey et al., 1997). In 1965, a study showed that in astronauts participating in orbital flights Gemini IV, V, and VII, a decrease in erythrocyte survival and in RBCM was occurring, due to erythrolysis of unknown cause (Fischer et al., 1967). On escaping the Earth's gravitational force the human organism experiences a reduction in total blood volume, plasma volume (PV) and, most importantly, of RBCM. The peripheral blood normally held in place by gravity, moves to central organs where a condition of acute plethora ensues. At the same time, a 20% reduction in PV takes place by redistribution in various compartments, thus inducing an increase in haematocrit ("pseudopolycythaemia") (Watenpaugh, 2001; De Santo et al., 2005). In the following days, a drop in EPO levels is observed, along with a decrease in RBCM of 10–15%, that has been likened to a phlebotomy of 700 ml of blood (**Figure 1**). According to the neocytolysis hypothesis, the latter decrease occurs too rapidly to be only the result of combined suppression of erythropoiesis and continued, normal destruction of physiologically aged RBCs (occurring at a rate of less than 1% per day), but could be explained by the selective lysis of relatively young RBCs, the "neocytes."

Neocytolysis would effect a finely-tuned and rapid regulation of the RBCM for a more efficient adaptation to mutated environmental conditions such as the following situations: in high-altitude dwellers (alpinists in mountaineering expeditions) returning to a normoxic environment at sea level; in anaemic uraemic patients requiring therapy with exogenous EPO (Rice et al., 1999); in patients with combined kidney and heart failure where a resistance to EPO treatment has been described to occur frequently (De Santo et al., 2005; van der Putten et al., 2008); and in a human model based on cycles of EPO injection and withdrawal (Rice and Alfrey, 2005). Moreover, the neocytolytic process could be relevant in other contexts as polycythaemia vera, blood-doping in sports, haemolytic anaemias (Mentzer et al., 1971), in patients with severe chronic obstructive pulmonary disease when subjected to oxygen therapy, where neocytolysis would occur upon therapeutic relieving of the hypoxic conditions (Harris and Epstein, 2001), and for the development of malaria anaemia, as recently proposed (Fernandez-Arias et al., 2013).

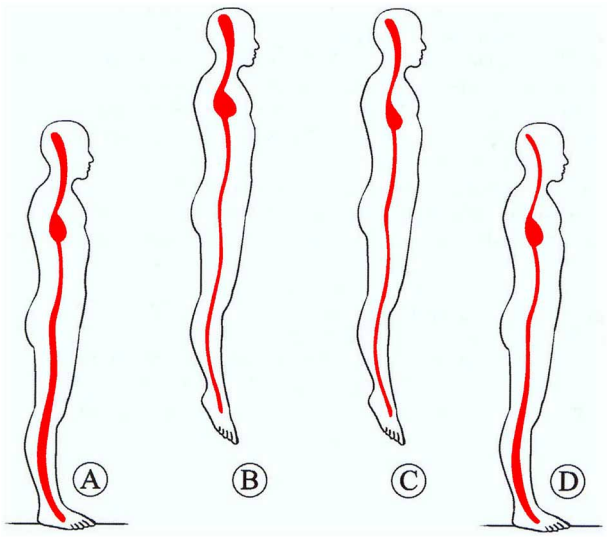

FIGURE 1 | Representation of central blood pooling in space. Upon transition from normogravity **(A)** to microgravity **(B)** the blood of the peripheral vascular space shifts to central space, causing central acute plethora, accompanied by peripheral vessel constriction. An adjustment is obtained by reduction of PV and RBC mass through erythrolysis and EPO reduction **(C)** over the first days of spaceflight. Then, upon return to normogravity **(D)**, the normal redistribution of blood volume and the augmentation of blood fluid reduce the haematocrit (space anemia). From Charles et al. (1994).

Comprehensive and stimulating reviews and commentaries have appeared that deal with neocytolysis, some from the same researchers who proposed the theory (Alfrey et al., 1997; Rice and Alfrey, 2000, 2005; Alfrey and Fishbane, 2007), some by others (Means, 1999; Harris and Epstein, 2001; De Santo et al., 2005; Handelman and Levin, 2010). We will limit this review to address some unresolved issues that in our opinion have not been sufficiently highlighted in previous works, and limit the focus on the experimental evidence obtained in space missions and high-altitude expeditions, the context where the theory was originally formulated, in particular in relation to the establishment of the link between EPO withdrawal and the removal of selected subpopulation(s) of RBCs.

EXPERIMENTAL EVIDENCE OF THE NEOCYTOLYTIC PROCESS IN SPACE MISSIONS

A decrease in RBCM of variable amounts was a consistent finding in astronauts during space flights. It was originally imputed to oxidation-induced haemolysis or oxygen inhibition of erythropoiesis, because an atmosphere of pure oxygen was breathed aboard of Gemini or Apollo missions. It was however observed also in subsequent missions where the atmosphere was similar to air at sea level, pointing therefore to a gravity-dependent mechanism capable of altering RBC survival. Evidence of erythrolysis under microgravity came from studies on Spacelab Life Sciences missions 1 and 2 (SLS-1 and SLS-2) (Udden et al., 1995; Alfrey et al., 1996a). Results showed unequivocally a reduction in RBCM, as measured, at landing, by radionuclide dilution

techniques with ^{51}Cr-labeled autologous RBCs and ^{125}I-iodinated albumin. When data from two other missions (Spacelab-1 and Space Transport System 41-B) were also considered, RBCM reduction was linearly correlated with mission length, and the extrapolation to day zero of flight suggested a faster RBCM decrease during the first days of flight (Alfrey et al., 1996a). On the first day of flight, PV also decreased by a significant 17%, with respect to values measured on 2 pre-flight days, thanks to the egress of albumin-containing fluid from the vascular space (the reasons for this are by no means clear, see Watenpaugh, 2001), and the establishment of a condition of "acute plethora" ensued (**Figure 1**).

Peripheral blood parameters revealed increased RBC concentration in-flight, of approximately 20%, which was related to the concomitant decrease in PV (Alfrey et al., 1996a). The microhaematocrit measured in-flight by centrifugation did not increase, despite the fall in PV, and this was explained with the observed concomitant decrease in MCV (calculated from RBC concentration and haematocrit). The explanation was that the number of young cells, which are larger, was decreasing. Crew members were sampled as early as day two in-flight, and every other day until flight day 14, but it is not clear how early the MCV decrease began (Alfrey et al., 1996a). The question is not trivial because it could help distinguish between a true selective removal of young RBCs and a generalized decrease in cell volume or the shrinkage of a subpopulation of cells, perhaps still, but not necessarily, the youngest circulating cells. To the best of our knowledge, no other study published or commented on MCV values measured during space missions, except for a decrease from pre-flight values of 90.9 ± 3.1 to 89.3 ± 2.8 fL (statistically significant despite the small difference) recorded at landing in 11 astronauts of the International Space Station Expeditions 1–8 (2000–2004) (Smith et al., 2005), but these were long-term missions (128–195 days), where compensatory processes appear to reset the homeostatic mechanisms and alleviate even the reduction in RBCM (Tavassoli, 1982).

The other important measured haematological parameter was EPO, whose levels decreased significantly, by about 25%, but only at days 2–4 in-flight, to return normal at flight days 8–12 (Alfrey et al., 1996a). In the previous SLS-1 study of three crew members, EPO levels were constantly 31% lower in-flight, but the mission only lasted 9 days (Udden et al., 1995).

The ferrokinetic data obtained by injecting ^{59}Fe 22 h or 72 h after launch for SLS-1 and SLS-2, respectively, and measuring the radiolabel in plasma and in RBCs, indicated that new RBC production in the bone marrow was not decreased from pre-flight values, and therefore this could not account for the magnitude of the decrease in RBCM (Alfrey et al., 1996b). New RBC production was not unexpected in SLS-1, where ^{59}Fe was injected after 22 h in-flight, because, despite the transient EPO decrease, committed erythroid precursors that were conceived before the labeling will continue to mature and will be released in the circulation during flight. For this reason, in SLS-2 radioiron was injected after 72 h in-flight. In this case, too, results indicated a normal RBC production. But SLS-2 is the mission where EPO levels returned to normal at flight day 8. Nonetheless, Authors concluded that the decrease in RBCM must have originated from an accelerated RBC destruction with respect to the physiological value (approximately 1%/day).

Reticulocyte counts (expressed as percent of RBCs) were only mentioned in the text for SLS-1, and were found to be decreased, for each crew member, with an average for all subjects of 0.6% on landing, after 9 flight days, with respect to 1% before the flight. Unfortunately, reticulocyte counts were not given for SLS-2, which would have allowed refining the definition of neocytes as including or not the reticulocytes (see below). Available data on reticulocytes in two Gemini VII astronauts showed no change between pre-flight values and landing and 2 days post-flight values. However, changes in haematological parameters and RBCM in Gemini missions were in part (and demonstrably) determined by hyperoxia conditions (Fischer et al., 1967; Tavassoli, 1982). Reticulocyte data are available also for the four crew members of the 10-day Spacelab 1 mission in 1984 (Leach and Johnson, 1984). Strangely, the reticulocyte counts were significantly lower, by approximately 50%, already the day before launch, with respect to pre-flight values (taken 1 week before flight), and, after an apparent return to values only 17% lower than baseline (non-significantly) at flight day 1, they were significantly 50% lower than baseline again at flight day 7. Interestingly, only the latter statistical significance was taken into account, whereas the former was disregarded as the result of "such factors as stress and previous blood withdrawals" (incidentally, previous blood withdrawals should increase, not increase reticulocyte counts) (Leach and Johnson, 1984). Additional results of this work showed that EPO levels did not change, and that RBCM decreased by about 1%/day, concluding that inhibition of erythropoiesis was not the only cause of RBCM reduction. Subsequently, however, EPO concentrations were correctly measured, with a newly available radioimmunoassay, in samples from the same mission and were found to be significantly decreased from flight day 1 until landing of that relatively short space mission (7 days) (Leach et al., 1988).

Other confounding factors in erythrokinetic studies include the ferritin status during and after space flight. Serum ferritin has been found increased after many long-term and short-term space missions (Smith et al., 2001), an indication of increased iron storage resulting from the destruction of RBCs (neocytes?) taking place during flight. However, it is well known that increases in serum ferritin may also derive from inflammatory or general stress response. To overcome this limitation, iron content in ferritin should be quantified. Measurements of this kind in astronauts returning from long-term missions have revealed a decrease in ferritin saturation despite the increase in serum ferritin (Smith et al., 2001, 2005). On the other hand, the concentration of circulating transferrin receptors (which decreases with iron load) was decreased in one study (Smith et al., 2001) but not significantly decreased in a more comprehensive subsequent report (Smith et al., 2005).

RANDOM LABEL METHOD WITH ^{51}Cr FOR EVALUATING RBC DESTRUCTION

Stronger evidence for an increased destruction of young RBCs in space came from the ^{51}Cr random labeling studies performed on six astronauts participating in the NASA shuttle missions SLS-1 and SLS-2. Here, autologous RBCs from six crew members from

each mission were labeled with ^{51}Cr and reinjected intravenously 21 days (SLS-1) or 12 days (SLS-2) before launch. Samples were then taken at intervals during the flight to determine the ^{51}Cr specific activity, i.e., the counts per minute per millilitre RBCs. The percent change in ^{51}Cr specific activity with respect to the value at time of injection was used as an estimate of RBC production and survival (Alfrey et al., 1996a).

Results showed that, after launch, the specific activity increased over that predicted had the astronauts remained on Earth. On landing day the mean difference was 6% more than predicted for the crew members, resulting from a much slower rate of change in specific activity in the first 4 days of flight with respect to that measured before flight (**Figure 2**). From data obtained in the SLS-2 mission the Authors concluded that the relative increase in ^{51}Cr specific activity was the result of a selective decrease in unlabeled cells, those produced in the days before flight, after the radiolabeling, and during flight, and not only the consequence of decreased erythropoiesis (due to the decrease in EPO) combined with normal RBC destruction rate. Interestingly, when data obtained in the SLS-1 mission were originally published (Udden et al., 1995), it was concluded that erythropoiesis was partially inhibited because the ferrokinetic study with ^{59}Fe revealed a lower incorporation of ^{59}Fe into RBCs and that the observed 11% decrease in RBCM was the result of suppressed release of RBCs into the circulation (it was speculated that EPO could have a role in permitting the egress of RBCs for the bone marrow) together with the normal age-related destruction of circulating RBCs at the physiological rate of 1%/day (Udden et al., 1995). In fact, in the SLS-2 mission the decrease in RBCM was more pronounced, and could not be explained only by the physiological rate of RBC removal and the relatively modest suppression of erythropoiesis (Alfrey et al., 1996a).

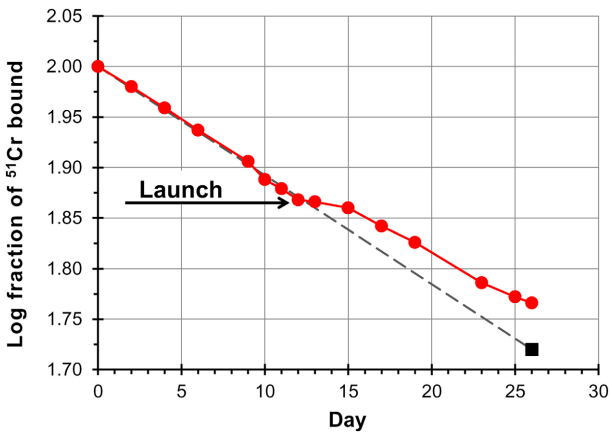

FIGURE 2 | Red cell survival on SLS-2 space mission. Data points are a composite of results from three astronauts. Red cell survival is normal pre-launch whereas the inflection in the curve beginning at launch has been interpreted as the result of destruction of unlabeled erythrocytes (neocytes), and a consequent increase in the concentration of labeled cells. The last point (square symbol) is based on the measured chromium remaining, corrected for the cell mass measured on landing day. The fact that the trend line (dashed line) generated from preflight values transects this point demonstrates that older labeled red cells are removed from the circulation at the same normal rate in space as on Earth. Redrawn from Rice and Alfrey (2005).

The rate of disappearance of the ^{51}Cr label from blood is determined by the rate at which new cells enter the circulation combined with the rate of elution of ^{51}Cr from the labeled RBCs. The method and the data obtained in space missions are valid only under the assumption that the rate of elution of ^{51}Cr from labeled RBCs is the same in space, under conditions of plethora and dramatic shifts in body fluids, as is on Earth. The Authors were conscious of this when they stated "If the rate of ^{51}Cr elution is assumed to be unaffected by spaceflight. . . " (Udden et al., 1995). However, as the rate of elution is known to vary amply, with an average of 1% per day under physiological conditions (Bentley et al., 1974), but with a much broader range of between 0.62 and 2.27% per day under pathological conditions (Cline and Berlin, 1963), one cannot but wonder what would be the situation under conditions of microgravity. In other words, a decrease in the steepness of the curves of ^{51}Cr disappearance from the circulation (Alfrey et al., 1996a) could be also explained by a decreased elution of the ^{51}Cr label taking place, for whatever reasons, as soon as the organism is exposed to microgravity conditions.

Another aspect that has not been sufficiently considered in the neocytolysis theory (but was contemplated in early studies, see Fischer et al., 1967) is the possible selective sequestration of young RBCs to other compartments, e.g., the spleen.

Before the advent of the neocytolysis hypothesis, interesting data on RBC survival were obtained from studies in rats aboard the Soviet Biosatellite Kosmos 782 and 936 (Leon et al., 1978, 1980). Here, a cohort-labeling method was used by feeding the animals ^{14}C-glycine 19 days before flight, and quantifying the respiratory ^{14}CO, derived from heme catabolism, starting at day 2 from landing. Unlike the random labeling with ^{51}Cr, this procedure is in principle immune from elution of the label, since only the ^{14}C biosynthetically incorporated into heme is measured (Landaw and Winchell, 1970). A number of parameters can be calculated, including the rate of random cell death and the rate of cell death by senescence (Leon et al., 1980). It turned out that the rate of random cell death of RBCs was higher in rats during space flight with respect to both a control group on Earth and, most notably, a group of rats that were exposed to simulated 1 g gravity on an on-board centrifuge. This was the first piece of evidence pointing to weightlessness as the cause of RBCM reduction, and excluding a number of possible factors such as hyperoxia, radiation, forces associated with launch and re-entry, temperature and humidity, diet. An increased rate of random destruction, and the consequent decrease in RBC death by senescence, together with the fluid shifts and central plethora (that may occur also in rats, as hypothesized, but not verified, by the Authors of this work) suggested on the one hand that RBCM reduction resulted from accelerated haemolysis, not only from suppressed erythropoiesis and normal destruction rate, and on the other, that the fluid shifts could change splenic function leading to increased sequestration and haemolysis in this organ (Leon et al., 1980). Another hypothesis, brought on by Tavassoli (1982) was that intramedullary haemolysis or selective lysis of reticulocytes (neocytes?) could explain the data of Leon et al. on rats. On the

other hand, random destruction, by definition, affects RBCs independently from cell age, and moves the focus on altered splenic function rather than specific cell properties. Because in these works on rats EPO concentrations were not monitored, an important parameter is missing, making it difficult to rationalize the data. Another important aspect is whether or not a plethora of the same magnitude as the one observed in humans also occurs in small animals exposed to microgravity (**Figure 3**).

A causal relationship was established (although only at a speculative level for the moment), between the disappearance of a young RBC subpopulation and the transient decrease in EPO observed during flight. It will be now examined on the basis of what experimental evidence this concept was extended to other models of physiological adaptive response and whether the causal relationship between neocytolysis and EPO decrease, which are so far only correlated events, was corroborated.

NEOCYTOLYSIS AFTER DESCENT FROM HIGH ALTITUDE

Since, more than 60 years ago, Merino (1950), analysing erythropoiesis in people living at high altitude (4540 m a.s.l.), described a decreased production and an increased destruction of RBCs during the deacclimatization at sea level, these observations remained obscure until 2001, when results of a study were published supporting the notion that neocytolysis is triggered also on descent from high altitude.

In this study (Rice et al., 2001), nine men living at 4380 m a.s.l. and polycythaemic (as expected for people living there) were analyzed for a baseline period of 11 days at high altitude and for additional 9 days after descent. Five blood samples were taken at altitude and four samples at sea level for analysis of various haematological parameters: haematocrit, haemoglobin concentration, reticulocytes, serum EPO, serum ferritin, serum transferrin receptor and heme isolation. In this study, to differentiate between young and old cells a cohort-labeling method, different from the ^{51}Cr random-labeling, was adopted. Participants ingested 1 g of ^{13}C-glycine on day 1 of the study and 1 g of ^{15}N-glycine on day 9. Blood and stool specimens were then analyzed for ^{13}C and ^{15}N enrichment in heme and stercobilin. Three participants were injected subcutaneously with 1200 U EPO daily beginning on the day of descent. Upon descent to sea level, serum EPO levels were decreased by approximately 80% in the six participants who did not receive exogenous EPO.

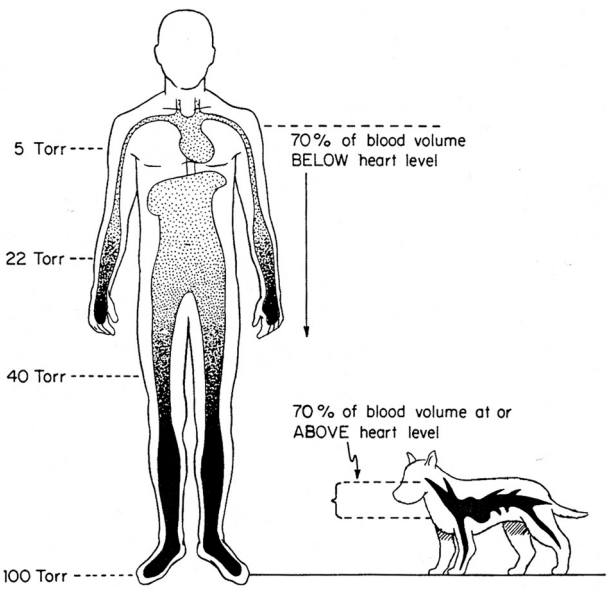

FIGURE 3 | Effects of terrestrial gravity on the cardiovascular system: the upright position. The impact of microgravity affects 70 and 30% of blood volume in humans and dogs, respectively. From Rowell (1983).

The average decrease in RBC mass was 7.0–9.6% within 3–7 days of descent. In the legend to the figure reporting those data, however, it is said that the measurements were performed "after 10 days at sea level." (Rice et al., 2001). It must be considered here that a decrease of RBCM of 7–9.6% in 7 days is close to the physiological value of RBC destruction under conditions where RBCs are not replaced by new ones (EPO levels decreased in these subjects by an average 80%, much more than in space missions). Concerning the isotopic enrichment in heme and stercobilin, the latter could not be determined because of low isotope levels, whereas the former was reported as being compatible with a decrease in young red cells at descent, although the differences were not statistically significant. Therefore, the main argument of the paper in favor of young RBC destruction was based on the observed elevation of ferritin levels, which was interpreted as the transfer of iron to stores. However, ferritin saturation was not measured, and this, combined with the observation that the levels of circulating transferrin receptors did not change, raises the suspicion that ferritin might have been elevated for other reasons (see above the discussion on these two parameters in space missions).

Another piece of evidence presented as a strong argument in favor of a haemolytic process triggered by descent to sea level was the observation that reticulocyte counts did not change with respect to baseline values ($120 \pm 52 \times 10^9$ cells/L at baseline vs. $141 \pm 52 \times 10^9$ cells/L during the first 6 days at sea level). Only on day 8 at sea level did the reticulocyte counts decrease to $90 \pm 54 \times 10^9$ cells/L, a delayed decrease that was correctly interpreted as the response to continued EPO suppression (remembering that reticulocytes released in the circulation at day 6 after descent were conceived in the bone marrow 6 days earlier in the presence of normal EPO levels). However, what is hard to reconcile with the neocytolysis hypothesis is that during the days when neocytolysis should be at its zenith, because plethora is at a maximum and EPO levels at the nadir, i.e., during the first week at sea level, the reticulocytes are spared by the process, as if they were something different from the neocytes. Yet, the Authors concluded: "that the decrease in red cell mass while reticulocyte production remained normal provides conclusive evidence of a haemolytic process" (Rice et al., 2001). Evidently, different features in the reticulocyte and neocyte must be invoked to accept this explanation, but a discussion about this aspect has never been conducted. Results of reticulocyte measurements in space missions (see above) and in descent from altitude are inconsistent and probably not informative of underlying changes in erythrokinetics. Mechanisms for the selective removal of RBCs produced under conditions of acutely enhanced erythropoiesis (stress reticulocytes) may also

be invoked (Landaw, 1988) but this explanation, while possibly applicable to RBCs produced at high altitude under hypoxic conditions, does not obviously work for space anaemia, where neocytes that were produced before flight (i.e., under normal, physiological conditions) are supposed to be destroyed.

It should be also mentioned results of a research where, after a 9 day training at 1900 m a.s.l. and return to sea level of eight elite endurance cyclists, total reticulocyte counts were slightly decreased, whereas the population of immature reticulocytes (detected as high- and medium- fluorescent cells in the analytical procedure) appeared to better reflect the changes in RBC production and destruction before and after descent, with a pattern compatible with an undergoing neocytolytic process (with all the limitations of the study, which include short acclimatization period, relatively modest altitude and high variability of the data) (Nadarajan et al., 2010).

EXPERIMENTS ON RBCs SUBSETS

The experiments conducted *in vivo*, through determination of haematological parameters on high altitude dwellers or astronauts gave conflicting and incomplete results, partially due to reasons inherent to this type of experiments, including the small number of subjects examined, which prevent any robust statistical analysis, and the environmental constraints the approach imposes. Blood withdrawal in space or at high altitude can be uneasy; furthermore, the storage of samples in space or at high altitude, with limited laboratory facilities, could alter the properties of the blood components. Finally, a further bias of the systemic approach is the non-univocal interpretation of some haematological parameters whose alteration can be due to reasons different from altered RBC mass and erythrolysis, as mentioned above.

In an attempt to somehow circumvent this problem, we tried to shift from a physiological, systemic approach to one at the cellular and molecular level, by analysing blood samples drawn before and after the exposure to microgravity or hypoxia, and by separating RBCs by density into age-related fractions (Risso et al., 2007, 2008).

A previous validation of the density separation procedure (Risso et al., 2007), and a characterization of some membrane components differentially expressed by old red cells, suggested that the neocytolysis hypothesis could be challenged through the investigation of the cell numbers and properties of the density separated subsets. Due to the technical constraints described above, it was not possible to conduct parallel measurements of 4.1a/4.1b ratio (see below), to verify whether changes in cell density that could occur on exposure to altered physiological conditions affected a selected subpopulation of RBCs.

In both the astronauts and the mountain climbers groups, after the exposure either to microgravity or high altitude, some of the standard haematological and cellular parameters (decrease of EPO plasma concentrations, increase in ferritin, decrease in reticulocytes) related to erythrolysis, indicated that this process was under way. In all subjects of both groups the percentage of low density (neocytes) after *vs.* before (control blood samples) the exposure to hypoxia or microgravity was reduced. The expression of membrane components which decline (CD55, CD47) or are translocated to the outer leaflet of the membrane bilayer (phosphatidylserine) in aged RBCs, and seem to be involved in the positive or negative regulation of phagocytosis, indicated that the less dense red cells (the few surviving neocytes) had a senescent phenotype, making them more prone to ingestion by macrophages (Risso et al., 2007, 2008).

In mountain climbers indeed a dramatic shift of the whole RBC population to high density regions of Percoll gradients seemed to indicate lysis also of many RBCs of the middle density (middle aged) subset and suggested a generalized increase in density of the whole RBC population, raising some doubts on the age-density relationship, at least under certain circumstances.

The observations on RBCs drawn from people adapted to high altitude were extended to other biochemical features. The ATP content of RBCs isolated during the deacclimatization from hypoxia was significantly higher than that of the control populations. Furthermore, alteration in the membrane-skeleton was found by a proteomic analysis, i.e., fragmentation of spectrin and actin (Risso et al., 2010). Finally in an investigation on blood samples drawn from mountain climbers, over and immediately after a 17 days' time spent at 3100–5600 m a.s.l., we observed that high altitude induced the expression of foetal haemoglobin. Gamma globin expression was detected in low but not in middle or high density RBCs, when separated from samples drawn during and after exposure to hypoxia (Risso et al., 2012). These observations, if on the one hand seem to suggest that also low density RBCs separated from particular blood samples can be reasonably regarded as a "neocyte-enriched" subset, on the other hand indicate that the "neocytolytic process" over high altitude could be related not only to a reduced EPO synthesis and to the need to decrease RBCs mass, but also to their particular features. Under the hypoxic stimulus, erythroid precursors likely generate RBCs which are endowed with biochemical features more fit to hypoxia and which, upon return to normoxia, should be cleared.

THE ROLE OF EPO

The role of EPO is controversial, in part for the reasons exposed above, because if one accepts the idea that the decrease in RBCM observed in microgravity and on descent from altitude can be accounted for only by the physiological destruction of 1% RBCs/day under conditions of decreased EPO, then there would not be an active, cytoprotective effect of EPO on circulating erythrocytes. The magnitude of RBCM deficit does however appear to be larger than expected at least for space missions of short duration (7–15 days), especially because EPO secretion is not suppressed, but only decreased, and only temporarily. The "space anaemia" condition is in fact described in haematology textbooks and treatises but it seems that neocytolysis is not yet received unanimously as the explanation for this phenomenon, because the fluctuations in EPO levels occurring during space flights are not considered sufficiently large to affect the RBCM (Erslev, 2001).

Conversely, RBCM decline after descent from altitude appears to be less dramatic, and compatible with a physiological rate of RBC destruction under conditions of strongly decreased EPO levels (see discussion above). It is tempting to speculate that the decrease in RBCM occurring in space and on descent from

altitude may only be apparently related to a common mechanism. At any rate, to account for RBCM decrease under both circumstances, a cytoprotective effect of EPO on circulating RBCs or RBC subpopulations has been invoked. The abrogation of any eryhrolytic process by EPO infusion in three mountain dwellers (the subjects remained polycythemic and no haematological parameter changes related to erythrolysis were observed), favors the hypothesis of an ongoing erythrolysis in the subjects that were not treated with EPO, and a possible causal correlation between EPO and lysis of (young) red cells (Rice et al., 2001), suggesting in that case a protective role played by EPO.

The existence of cytoprotective effects of this haematopoietic hormone on a variety of different cell types has been described (Kowalczyk et al., 2011; Shin et al., 2012). A study of the complex expression pattern of EPO receptors in primary human erythroid precursors induced to differentiate to reticulocytes, revealed the lack of EPO receptors, at least in these *in vitro*-differentiated reticulocytes (Sawada et al., 1990; Broudy et al., 1991; Wickrema et al., 1992). Mature erythrocytes were found to virtually lack EPO receptors, although murine RBCs, specifically the 2% youngest (less dense) RBCs, were found to express them (Mihov et al., 2009). In human erythrocytes, EPO has been described to inhibit a Ca^{2+}-permeable cation channel, whose opening can be evoked *in vitro* by hyperosmotic shock, via a mechanism implying the binding of EPO to EPO receptors that were found to be present, as ^{125}I-EPO-binding sites, in an average number of six per cell (Myssina et al., 2003). It must be observed, in the light of recent evidence (Minetti et al., 2013), that care must be taken in avoiding artefacts originated by contaminating granulocytes in RBC preparations, especially when a possible enrichment in contaminating cells is produced when RBCs are sub-fractionated according to density.

At systemic level, pleiotropic effects of EPO acting not only as a haemopoietic hormone but also in the regulation of PV, in interplay with the renin–angiotensin–aldosterone axis, have been reported (Lundby et al., 2007). On the other hand, EPO effects seem to be context-dependent, as EPO produced in brain, liver, spleen, lung and testis (where EPO mRNA was detected) is unable to substitute for renal EPO in chronic kidney disease, and brain EPO seems to act locally as neuroprotector (Jelkmann, 2011). Other difficulties in accepting direct effects of EPO, mediated by its receptor, in non-erythroid tissues include evidence that after suppressing EPO receptor expression in all organs except bone marrow in mice, normal and fertile animals develop (Suzuki et al., 2002), and that EPO receptors are undetectable in non-haematopoietic cells (Sinclair et al., 2010; Jelkmann, 2011). On the other hand, it was shown that erythropoietically inactive EPO derivatives act as cytoprotective factors in animal models (Leist et al., 2004). Would it be possible that EPO act, by molecular mimicry, on a different receptor system in other cell types, including the RBC? Against this possibility is evidence that the increased viability of peripheral RBCs measured with the ^{51}Cr random labeling technique in uraemic patients on recombinant human EPO treatment was ascribed to molecular effects of EPO on the erythroid precursor cells in the bone marrow, and not on peripheral circulating erythrocytes (Polenakovic and Sikole, 1996).

Because of the inevitable scepticism with which claims of the expression of EPO receptors in erythrocytes are met, it could be hypothesized that the observed effect of EPO may be due to the interference of EPO with other molecular targets in RBCs.

CONCLUSIONS

Neocytolysis is a physiological process which could shorten RBCs lifespan in response to a changed external environment and lead to a reduction in RBC mass. Despite many studies, the factors determining the lifespan of cells (including RBCs) that circulate in blood are not fully understood. In the past years, in red cells treated *in vitro* with some pro-apoptotic agents, a programmed cell death-like process has been described, which has been dubbed eryptosis (Lang et al., 2008). Investigations on the features and events associated with eryptosis have shown that they are reminiscent of those already described in old senescent red cells (dehydration, cell shrinkage, increase in cell density) and of those observed in nucleated apoptotic cells [PS exposure, increased intracellular Ca^{2+} concentration, altered functionality of ion channels, see Lang et al. (2012)]. Then it appears that, under some circumstances, RBCs death could be due to eryptosis.

Since RBCs lack the organelles and the multienzymatic, biogenic machineries able to protect the cells from external injuries, they are particularly sensitive to any changes either of inner proteins (altered haemoglobin, membrane lipid peroxidation, alteration of membrane-skeleton) or of external signals perturbing their homeostasis.

Within this framework, a shortening of lifespan is conceivable whenever changes in haemoglobin (for instance, in thalassemia or sickle cell disease) lead to alterations in membrane-skeleton, cell shape, redox conditions, or changes in the external environment requiring a fast reduction of RBCM, speed up the programmed death (or senescence process), followed by macrophage phagocytosis.

In this latter case, while reduction of RBCM has been documented in both exposure to microgravity and hypoxia, and some data seem to indicate that erythrolysis is not at random, two main issues need more detailed investigation: 1, the concomitant decrease in EPO levels and RBCM, since, although a causal link could be (and it has been) hypothesized, a formal evidence of this relation is still lacking; and 2, the identification of the targeted red cells. In relation to the latter argument, although the studies on age-related subsets seem to indicate that the low- / middle-density RBCs could be prone to phagocytosis, in view of their "eryptotic" (or "senescent") phenotype, the relation between low density and age on the RBCs population after exposure to hypoxia or microgravity is questionable.

Neocytolysis is a fascinating hypothesis that, for the reasons exposed here, should be subjected to further scrutiny. It would benefit from being tested with additional methods that are immune from artefacts possibly arising from the different conditions existing on Earth and under microgravity. One such approach would be to conduct systematic and accurate measurements of the RBC age parameter 4.1a/4.1b, which is an absolute marker of RBC age independent on cell density, metabolic activity or imponderable side effects of radiolabeling. It is a molecular clock (Robinson and Robinson, 2004) whose ticking

is only dependent on temperature, and therefore is relatively stable in a homeothermic animal. Measurements of 4.1a/4.1b could be performed on the total population of circulating RBCs or on cells separated by density. This approach yielded interesting results when applied to the study of how cell age impacts on RBC stored under blood bank conditions (Minetti et al., 2001). Moreover, a more refined and rigorous definition of the neocyte is required, because the reticulocytes are sometimes comprised in this definition (space missions in general) and sometimes not (descent form altitude). The difficulty in discovering (Chang et al., 2009) molecular species in neocytes which could mediate the response to decreased EPO levels, leading to recognition and destruction of young RBCs, may be a symptom of the extremely elusive nature of these species, depend on the necessity of implementing a multicellular *in vitro* model (Trial et al., 2001; Trial and Rice, 2004), suggest to look for off-target effects of EPO (Jelkmann, 2011), or indicate the very absence of such molecular machinery.

It would be worth restarting from what we know about RBCs of different age. For instance, how the intracellular ion homeostasis and membrane permeability are regulated in response to microgravity. Nothing is known of the calcium content or permeability of RBCs (Bogdanova et al., 2013) in space. Young RBCs have lower sodium and higher potassium and water content per-cell than older RBCs. There are several pathways that can be activated and determine potassium efflux (Bernhardt and Weiss, 2003). This could result in cell shrinkage, associated or not with membrane vesiculation (Ciana et al., 2004; Willekens et al., 2008), that could lead to reduction in deformability and the appearance of a senescent phenotype in otherwise young RBCs. A poorly deformable, dehydrated young erythrocyte would spend more time in the spleen sinusoids, where it could be recognized as abnormal and sequestered by the splenic macrophages. This is the rationale for the indication of splenectomy to increase RBC survival in some pathological conditions. "Nothing is known about splenic function in space" (Tavassoli, 1982), and it would be interesting to verify whether neocytolysis could take place also in splenectomized subjects. The possible occurrence of young RBC shrinkage under microgravity is compatible with the observation of the rapid decrease in MCV occurring in the 1st h of flight (see above). On the other hand, no study has ever been conducted on the impact of microgravity (in space or simulated) on RBC membrane permeability (Prof. Dr. Ingolf Bernhardt, personal communication).

Density separation of RBCs must be accompanied by determination of an absolute marker of cell age such as 4.1a/4.1b ratio. This will help clarify our own results obtained in mountain climbers after descent to sea level, where a massive shift in density profiles of RBCs from low to high density regions of Percoll gradients were observed (Risso et al., 2007).

Combined measurements of 4.1a/4.1b ratio and density separation of RBCs could also shed light on such discrepancies as the unclear behavior of reticulocyte counts, which were described to decrease during flight, but were also found 50% lower the day before flight (Leach and Johnson, 1984). Reticulocytes are probably not reliable indicators of the real size of the population of young circulating RBCs.

Further studies are needed to establish whether the reduction in RBCM, which is an established fact in a variety of blood disorders or physiological adaptive responses, is due, in each condition, to the removal of a selected population of RBCs, whether this is a population of "neocytes," and what are the features of the targeted cells.

Given the complexity of the systemic responses, space anemia, uraemic anemia and deacclimatization anemia could be unrelated processes altogether. Each should be re-examined in its own context before generalizations could be made.

AUTHOR CONTRIBUTIONS

Angela Risso conceived the review, conducted literature survey and wrote the first draft; Annarita Ciana and Cesare Achilli conducted literature survey, contributed to the introduction section and reviewed all other parts, Guglielmo Antonutto contributed to the literature survey and to discussions on the physiological issues of human haemodynamic and of red cells in space, Giampaolo Minetti conducted literature survey, wrote subsequent versions and overviewed the writing process.

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Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging

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Red Blood Cells (RBCs) need to deform and squeeze through narrow capillaries. Decreased deformability of RBCs is, therefore, one of the factors that can contribute to the elimination of aged or damaged RBCs from the circulation. This process can also cause impaired oxygen delivery, which contributes to the pathology of a number of diseases. Studies from our laboratory have shown that oxidative stress plays a significant role in damaging the RBC membrane and impairing its deformability. RBCs are continuously exposed to both endogenous and exogenous sources of reactive oxygen species (ROS) like superoxide and hydrogen peroxide (H_2O_2). The bulk of the ROS are neutralized by the RBC antioxidant system consisting of both non-enzymatic and enzymatic antioxidants including catalase, glutathione peroxidase and peroxiredoxin-2. However, the autoxidation of hemoglobin (Hb) bound to the membrane is relatively inaccessible to the predominantly cytosolic RBC antioxidant system. This inaccessibility becomes more pronounced under hypoxic conditions when Hb is partially oxygenated, resulting in an increased rate of autoxidation and increased affinity for the RBC membrane. We have shown that a fraction of peroxyredoxin-2 present on the RBC membrane may play a major role in neutralizing these ROS. H_2O_2 that is not neutralized by the RBC antioxidant system can react with the heme producing fluorescent heme degradation products (HDPs). We have used the level of these HDP as a measure of RBC oxidative Stress. Increased levels of HDP are detected during cellular aging and various diseases. The negative correlation ($p < 0.0001$) between the level of HDP and RBC deformability establishes a contribution of RBC oxidative stress to impaired deformability and cellular stiffness. While decreased deformability contributes to the removal of RBCs from the circulation, oxidative stress also contributes to the uptake of RBCs by macrophages, which plays a major role in the removal of RBCs from circulation. The contribution of oxidative stress to the removal of RBCs by macrophages involves caspase-3 activation, which requires oxidative stress. RBC oxidative stress, therefore, plays a significant role in inducing RBC aging.

Keywords: red blood cells, oxidative Stress, deformability, heme degradation, cellular aging

INTRODUCTION

THE RED BLOOD CELL OXIDATIVE STRESS

The functional role of Red Blood Cells (RBCs) is the transport of oxygen from the lungs to the tissues providing all cells with the required oxygen. In the circulation, RBCs are continuously exposed to both endogenous and exogenous sources of reactive oxygen species (ROS) that can damage the RBC and impair its function. To minimize the effect of these ROS and the resultant oxidative stress, RBCs have an extensive antioxidant system involving both non-enzymatic low molecular weight antioxidants like glutathione and ascorbic acid and enzymatic antioxidants including superoxide dismutase, catalase (Gonzales et al., 1984), glutathione peroxidase (Nagababu et al., 2003) and peroxiredoxin-2 (PRDX-2) (Lee et al., 2003; Nagababu et al., 2013).

ROS are highly reactive and many of the ROS released from neutrophils and macrophages into the plasma are neutralized before they can be taken up by RBCs. However, particularly in the microcirculation, where the RBCs are in close proximity and even make contact with the vasculature (Rifkind et al., 1997; Nagababu and Rifkind, 1998), ROS released from neutrophils (Aoshiba et al., 1999), macrophages and endothelial cells are taken up by RBCs. Upon entry into RBC cytoplasm, they are for the most part neutralized by the cytosolic antioxidant system. In fact, hydrogen peroxide added to RBCs rapidly reacts with catalase being converted to oxygen without any oxidation of hemoglobin (Hb). In addition, endogenous ROS are continuously generated by the slow autoxidation of Hb (Abugo and Rifkind, 1994), which produces methemoglobin (that can no longer carry oxygen) and superoxide that rapidly dismutates to form hydrogen peroxide. The bulk of these ROS are also neutralized by the RBC cytosolic antioxidants. However, the ability of the antioxidant system to neutralize the endogenous ROS is limited as the blood flows through the microcirculation and Hb becomes partially oxygenated. Partial oxygenation results in a Hb conformational change with certain unique properties (see below). Thus, there is a dramatic increase in the rate of Hb autoxidation for partially oxygenated Hb (Abugo and Rifkind, 1994; Balagopalakrishna et al.,

1996). At the same time an increase in the affinity of partially oxygenated Hb for the RBC membrane (that is appreciably greater than for fully oxygenated or fully deoxygenated Hb (Cao et al., 2009), limits the efficiency of the antioxidant system (that is primarily cytosolic) from neutralizing the ROS formed at the membrane. This pool of un-neutralized ROS in the RBC has been shown to (1) damage the RBC membrane (Nagababu et al., 2008; Barodka et al., 2013b) impairing the flow of RBCs through the microcirculation and the delivery of oxygen to the tissues and to (2) be transferred to cells, which come in contact with RBCs resulting in tissue damage that induces inflammation (Kiefmann et al., 2008; Huertas et al., 2013).

Recent studies indicate that RBCs also contain NADH oxidases, which can generate endogenous ROS (George et al., 2013). The initial demonstration of RBC NADH oxidase and its potential physiological ramification involved sickle cell disease. However, some forms of NADH oxidase were also detected in normal RBCs. Additional studies are necessary to determine the functional effect of NADH oxidase in normal cells and any possible effect on cellular aging. As found for Hb generated ROS, the ROS generated in the cytoplasm would under most conditions be neutralized by the RBC antioxidant system. However, George et al has found that one of the NADH oxidase isoforms present in RBCs is located on the membrane. The potential effect of this isoform of NADH oxidase needs to be further investigated during cellular aging in normal RBCs.

THE LEVEL OF HEME DEGRADATION, A MEASURE OF RBC OXIDATIVE STRESS

The presence of antioxidant enzymes as well as the relative instability of ROS makes it very difficult to quantitate the pool of un-neutralized ROS that reflect RBC oxidative stress. This affects both the RBC and other cells the RBC comes in contact with. As a solution for this problem we have found that a small fraction of the non-neutralized hydrogen peroxide degrades the protoporphyrin producing fluorescent heme degradation products (HDPs) that can be detected even at very low concentrations (Nagababu and Rifkind, 1998). These HDPs are also not neutralized by the RBC antioxidant systems and are, therefore, much more stable.

These HDPs were originally detected (Nagababu and Rifkind, 1998) when a 10 fold excess of hydrogen peroxide was added to oxyhemoglobin (oxyHb). At this concentration, in addition to the formation of methemoglobin (metHb), ∼5% of the hemes were degraded producing two fluorescent products. One of those has an excitation wavelength of 321 nm and emission wavelength in the region of 465 nm and the second product has an excitation wavelength of 460 nm and emission wavelength in the region of 525 nm. Confirmation that these fluorescent bands are attributed to HDPs is based on the observation that the same fluorescent bands were obtained when hydrogen peroxide reacted with heme or hemin, although these reactions required much higher levels of hydrogen peroxide. In addition, the excitation and emission wavelengths for these bands were distinct from those of globin fluorescent amino acids like tryptophan, tyrosine or di-tyrosine (Teale, 1960; Giulivi and Davies, 1993) as well as free protoporphyrin IX. Thus, these fluorescent bands originating from heme degradation are considered as markers of RBC oxidative stress.

The mechanism for the formation of these degradation products was shown to require (Nagababu and Rifkind, 2000) an initial reaction with hydrogen peroxide producing Fe(IV) ferrylhemoglobin (ferrylHb). The formation of ferrylHb was confirmed by showing that sodium sulfide, which reacts with ferrylHb, inhibits the formation of the HDPs. FerrylHb then reacted with a second molecule of hydrogen peroxide. The requirement for this hydrogen peroxide was demonstrated by the finding that catalase added after the ferrylHb had formed, inhibited the formation of HDPs. This second molecule of hydrogen peroxide produced metHb and a superoxide radical, which was retained in the heme pocket and was detected by electron paramagnetic resonance. The retention of this superoxide in the heme pocket much longer than the superoxide formed during Hb autoxidation (see above) facilitates a reaction of the superoxide with the porphyrin initiating the heme degradation process.

The significance of this reaction is indicated by the demonstration that the same HDPs are generated from the low levels of hydrogen peroxide constantly being produced by the dismutation of superoxide released (Nagababu and Rifkind, 2000) during the autoxidation of purified Hb.

In studies with intact RBCs, we found that we can detect the same fluorescent band (Ex: 321 nm) as in HDPs described above in any fresh RBC sample (Nagababu et al., 2010). We further demonstrated that the amount of heme degradation increased for RBCs in circulation for a longer period of time (older RBCs) (Nagababu and Rifkind, 2004). These results indicate that HDPs are produced in the RBCs even though they have an extensive antioxidant system that should react with any amount of hydrogen peroxide formed.

This paradox is explained by the finding that in RBCs, almost all of the HDPs are located on the membrane (Nagababu et al., 2010). To rule out the uptake of cytoplasmic HDPs by the more hydrophobic membrane, we incubated Hb reacted with hydrogen peroxide with RBC membranes and found no increase in the level of membrane fluorescent products over a period of 12 h. These results thus indicate that the HDPs generated in the RBC are formed on the RBC membrane and not in the cytoplasm.

Hb is known to bind to the cytoplasmic end of band 3 (Evans and Fung, 1972; Shaklai et al., 1977a,b) present in the membrane. It has been documented that deoxyhemoglobin (deoxyHb) has an appreciably higher affinity for band 3 than oxyHb. This difference has been attributed to changes in the subunit interactions, which facilitate interactions between the cytoplasmic end of band 3 and Hb. While these earlier studies have compared fully oxygenated and fully deoxygenated Hb with known differences in quaternary structure, we have been involved in the studies with partially oxygenated Hb present in RBCs in the microcirculation. Evidence for a distinct conformation for partially oxygenated Hb was initially demonstrated by the dramatic increase in the rates of autoxidation when Hb is partially oxygenated (Abugo and Rifkind, 1994; Balagopalakrishna et al., 1996). Recent studies (Cao et al., 2009) imply that this same conformational change, which alters the interactions between Hb subunits, also has a dramatic effect on their affinity for the RBC membrane. We, thus,

found that low levels of nitrite/NO reacted Hb present in fully deoxygenated RBCs have an affinity >100-fold greater for the RBC membrane than deoxyHb. We have attributed this to the nitrite/NO bound fraction of the Hb that is partially liganded, with properties similar to that of partially oxygenated Hb.

Thus, the partially oxygenated Hb is responsible for the bulk of the ROS formed by Hb autoxidation. However, with the elevated affinity of this fraction of Hb for the RBC membrane, the superoxide and hydrogen peroxide formed during the autoxidation of this Hb, is relatively inaccessible to the cytosolic catalase and superoxide dismutase. So, an appreciable fraction of these ROS can react with the Hb before being neutralized by the RBC antioxidant system. Unlike the potential for such reactions with endogenously generated ROS, the addition of exogenous hydrogen peroxide is immediately transported into the RBC before it can react with membrane bound Hb and is neutralized predominantly by cytosolic catalase (Nagababu et al., 2010). While catalase does not seem to be able to compete with Hb in reacting with the pool of hydrogen peroxide generated on the membrane, glutathione peroxidase (Nagababu et al., 2003) and PRDX-2 (Nagababu et al., 2013) may play a role in neutralizing ROS generated on the RBC membrane. Glutathione peroxidase is known to react with membrane ROS (Horton and Fairhurst, 1987) and its inhibition was found to dramatically increase the formation of HDPs. Although PRDX-2 is primarily a cytosolic enzyme, ∼5% of it is membrane associated (Moore et al., 1991; Low et al., 2004). The neutralization of membrane generated ROS by PRDX-2 was postulated to explain an increase in heme degradation in PRDX-2 knockout mice (Nagababu et al., 2013).

RBC OXIDATIVE STRESS IMPAIRS CELLULAR DEFORMABILITY NECESSARY FOR EFFECTIVE OXYGEN TRANSPORT AND DELIVERY

RBCs are larger than the capillary diameter in the microcirculation (Pries et al., 1996). Blood flow, therefore, requires that the discoid RBCs deform to squeeze through these capillaries and deliver oxygen to the tissues. The ROS generated on the RBC membrane through Hb autoxidation are ideally located to react with membrane lipids and proteins producing lipid peroxidation and modified membrane proteins that can affect the membrane structure.

Exogenous xanthine oxidase, which generates superoxide, has been shown to affect the lipids by increasing their peroxidation. It has, however, been shown that this lipid oxidative damage does not affect the RBC deformability (Gurbuz et al., 2004). Nevertheless, it has been shown that deformability of RBCs is impaired as a result of treatment of RBCs by a number of reagents associated with oxidative stress including hydrogen peroxide, t-butyl hydroperopxide, cumene hydroperoxide, verapamil and ascorbate (Kuypers et al., 1990; Kim et al., 2008). Damage to membrane proteins is, thus, presumably responsible for the impaired cellular deformability associated with oxidative stress. The role of protein damage in producing impaired deformability is consistent with a dominant role for the membrane cytoskeleton (Suzuki et al., 2007) in regulating RBC deformability. There are a number of specific cases where it has been shown that damage to membrane and cytoskeletal proteins affects deformability (Chasis and Mohandas, 1986; Cluitmans et al., 2012; Grau et al., 2013).

A linear relationship between deformability and RBC oxidative stress, as measured by the level of HDPs (see above) has been found in studies involving sickle cell disease where subjects under crisis and not under crisis as well as those with sickle cell trait were compared (Barodka et al., 2013b). Comparing all of these subjects, a highly significant correlation [$N = 34$; $R = (-)0.68$; $p < 0.0001$] was found between RBC deformability and the level of HDPs. We also found that with transgenic mice lacking PRDX-2 and superoxidase-2 (SOD2) (Mohanty et al., 2013; Nagababu et al., 2013), there were appreciable increases in heme degradation that coincided with a decrease in RBC deformability.

Despite this correlation, by comparing the average changes in deformability and heme degradation for normal healthy subjects, subjects with sickle cell trait, subjects with sickle cell disease not undergoing crisis and those undergoing crisis, we were able to delineate the different factors that contribute to the changes in heme degradation and RBC deformability. Heme degradation, which is primarily affected by the instability of the Hb, increased even for subjects with sickle cell trait, because of the increase in unstable sickle cell Hb. On the other hand, the RBC deformability in addition to being affected by oxidative stress was also affected by the changes in Hb aggregation that occurs during sickle cell crisis (see below).

The contribution of oxidative stress that does not necessarily involve Hb autoxidation to deformability is indicated by caspase-3. Caspase 3, is activated in the RBC by oxidative reactions, such as the reaction with tertiary butyl hydroperoxide, has been shown to partially degrade band 3 (Mandal et al., 2003; Clementi et al., 2007). This reaction has been shown to induce the exposure of phosphatidylserine (PS, usually located on the inner leaflet of the RBC membrane) to the outer surface (Mandal et al., 2005). This dramatic rearrangement of the membrane has been shown to involve a concomitant decrease in deformability (Fens et al., 2012).

Oxidative stress has also been shown to inhibit Ca-ATPase (Samaja et al., 1990; Kiefer and Snyder, 2000), which is responsible for limiting the intracellular concentration of calcium. Enhanced intracellular calcium has several effects that can affect deformability. This includes activation of the Gardos channel resulting in leakage of potassium from the RBC affecting cation homeostasis (Ney et al., 1990; Barodka et al., 2013a) causing shrinkage of the cell and impaired deformability. Calcium also activates calpain that can degrade additional proteins on the membrane (Redding et al., 1991).

Despite the demonstrated relationship between RBC deformability and oxidative stress, deformability can be affected by other processes that are not associated with oxidative stress. Although calcium induced shrinkage is associated with oxidative stress, membrane microvesiculation is a regulated process that is accelerated in older cells (Willekens et al., 2008) and is not thought to involve oxidative stress. It is, however, responsible for the increase in cell density coupled with a decrease in the cellular deformability and flexibility (Bartosz, 1991; Abugo and Rifkind, 1994; Wang et al., 2010). In addition to regulated vesiculation, any change that affects the volume of the cell and the excess surface area, will affect the deformability of the RBCs.

There are, in addition, changes in the intracellular content that can affect deformability independent of oxidative stress. A documented case of such a deformability change involves sickle cell disease (Barodka et al., 2013b). We have alluded to the correlation between heme degradation and impaired deformability for sickle cell disease which is attributed to the instability of sickle cell Hb (see above). However, a careful analysis of the data indicates that deformability is also affected by the aggregation of Hb that involves the structural change in deoxygenated Hb for sickle cell Hb, which triggers sickling of the RBC during sickle cell crisis. In this case the sickling process directly impairs the ability of the cell to deform.

CONTRIBUTION OF RBC OXIDATIVE STRESS TO RBC AGING

The RBC, continuously undergoing normoxic and hypoxic cycling, is constantly exposed to oxidative insults during its 120 day life-span that results in continuous biochemical, physical, and structural changes. These changes impair the ability of the RBC to transport oxygen and eventually trigger its removal from the circulation by the reticulo-endothelial system. The reticulo-endothelial system involves the mononuclear phagocytic cells primarily in the spleen, but also in the liver and lymph nodes.

The processes responsible for the actual triggering of the removal have been extensively studied (Ajmani and Rifkind, 1998; Barvitenko et al., 2005; Rogers et al., 2009; Antonelou et al., 2010). Many of the processes involve oxidative stress.

The RBC membrane band 3 is the dominant integral transmembrane protein. It has several crucial functions including: (1) the maintenance of anion homeostasis, (2) providing a link between the membrane and the cytoskeleton responsible for maintaining the cell shape and (3) providing for the interaction of a number of cytosolic proteins with the membrane via the amino terminal region that protrudes into the cytosol. This region of band 3 binds competitively both Hb, and a number of glycolytic enzymes (Mohandas and Gallagher, 2008). The changes in Hb binding to band 3 as a function of the Hb oxygenation, therefore, couple Hb oxygenation, Hb autoxidation, glycolysis and ATP production (De Rosa et al., 2008).

Oxidative damage to band 3 has been linked to RBC aging including the exposure of senescent specific neo-antigens that bind autologous IgG triggering RBC removal (Kay, 1993). IgG binding has also been linked to band 3 clusters, which is triggered by the binding of denatured oxidized Hb (hemichromes) to band 3 (Low et al., 1985; Rettig et al., 1999; Ferru et al., 2011).

Caspase-3 activation, which involves oxidative stress (see above), also cleaves the cytoplasmic end of band 3 (Mandal et al., 2003) affecting the interactions of band 3 with cytosolic proteins as well as the linkage to ankyrin and the cytoskeleton, which also induces PS exposure (Grey et al., 2012) (see below).

Membrane micro-vesiculation is a process that accelerates in the formation of older cells (Willekens et al., 2008) (see above). These changes limit the ability of the RBC to maintain the highly deformable biconcave shape necessary to pass through narrow pores, thus contributing to their removal from circulation. While cell shrinkage and vesiculation can be induced by various factors, some of which may not involve oxidative stress, the shrinkage associated with potassium leakage through the Gardos channel is triggered by oxidative stress. This process is initiated by damage to Ca-ATPase, which maintains a low intracellular concentration of free calcium ions (Larsen et al., 1981). Damage to Ca-ATPase is responsible for the age induced increase in intracellular calcium and is generated by oxidative damage to the ATPase (Samaja et al., 1990; Kiefer and Snyder, 2000). The increase in intracellular calcium activates the Gardos channel causing the leakage of potassium from the cell resulting in cell shrinkage and impaired deformability (Brugnara, 1993; Foller et al., 2008b).

An increase in intracellular calcium also activates calpain, transglutaminase-2 and some caspases that can degrade/crosslink cytoskeleton proteins (Redding et al., 1991). It also inhibits phosphotyrosine phosphatase increasing band 3 phosphorylation (Zipser et al., 2002).

The RBC lipid bilayer contains an asymmetric distribution of phospholipids with PS being maintained on the inner surface of the membrane by the competition between Scramblase, which randomizes the distribution and Flippase, which internalizes the PS. Coupled with an increase in Sphingomyelinase that increases ceramide, increased intracellular calcium has been linked to the exposure of PS and to a reduction in Flippase activity (Burger et al., 2013), that triggers the interaction of RBCs with macrophages and eryptosis (Daleke, 2008; Foller et al., 2008a; Weiss et al., 2011). Despite the important role of macrophages in the removal of RBCs,it is not clear that the interaction of RBCs with macrophages is responsible for the removal of aged RBCs from circulation (Dasgupta et al., 2008; Saxena et al., 2012).

CONCLUSION

Partially oxygenated Hb molecules formed in the RBCs in microcirculation, when oxygen is being transported by them to the tissues, have an elevated affinity for the RBC membrane and have an increase in autoxidation producing ROS that are not completely neutralized by the RBC antioxidant system. This source of RBC oxidative stress is involved in a number of the factors that contribute to RBC aging and the removal of RBCs from the circulation. This oxidative process, thus, explains the dominant role of oxidative stress in RBC aging.

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The involvement of cation leaks in the storage lesion of red blood cells

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Stored blood components are a critical life-saving tool provided to patients by health services worldwide. Red cells may be stored for up to 42 days, allowing for efficient blood bank inventory management, but with prolonged storage comes an unwanted side-effect known as the "storage lesion", which has been implicated in poorer patient outcomes. This lesion is comprised of a number of processes that are inter-dependent. Metabolic changes include a reduction in glycolysis and ATP production after the first week of storage. This leads to an accumulation of lactate and drop in pH. Longer term damage may be done by the consequent reduction in anti-oxidant enzymes, which contributes to protein and lipid oxidation via reactive oxygen species. The oxidative damage to the cytoskeleton and membrane is involved in increased vesiculation and loss of cation gradients across the membrane. The irreversible damage caused by extensive membrane loss via vesiculation alongside dehydration is likely to result in immediate splenic sequestration of these dense, spherocytic cells. Although often overlooked in the literature, the loss of the cation gradient in stored cells will be considered in more depth in this review as well as the possible effects it may have on other elements of the storage lesion. It has now become clear that blood donors can exhibit quite large variations in the properties of their red cells, including microvesicle production and the rate of cation leak. The implications for the quality of stored red cells from such donors is discussed.

Keywords: red blood cell storage, storage lesion, cation leak, vesiculation, oxidation, transfusion

INTRODUCTION

Stored red blood cells (RBCs) have provided life-saving transfusions for many years. Over this time improvements in storage techniques and media have increased the viability and functionality of stored RBCs. The development of storage solutions (reviewed in Moore, 1987; Hess, 2006) citrate-phosphate-dextrose (CPD) and acid-citrate-dextrose (ACD) (Weisert and Jeremic, 1973), saline-adenine-glucose (SAG) (Ambrus et al., 1975; Kreuger et al., 1975; Herve et al., 1980; Strauss et al., 1980; Peck et al., 1981), with added mannitol (Högman et al., 1983) as in the SAG-M additive commonly used in the UK today, or the more recent development of chloride-free additives (Högman et al., 2006) and phosphate-adenine-glucose-guanosine-gluconate-mannitol (PAGGGM) (Burger et al., 2010), has helped to preserve metabolic functions and reduce lipid peroxidation (Knight et al., 1993). Leukoreduction, which was introduced in the UK in the late 1990s (AuBuchon et al., 1997; reviewed in Roddie et al., 2000) primarily to reduce the transmission of viruses, was shown to decrease hemolysis (Williamson et al., 1999) and the oxidative damage and calcium-related stress of stored RBCs (Antonelou et al., 2012). Nonetheless, stored RBCs still deteriorate during storage in ways that are not fully understood (Hess, 2012) and this "storage lesion" has been implicated in the poor outcome, post-transfusion, of certain categories of patients (Wang et al., 2012).

In the UK RBCs may be stored for 35 days prior to transfusion. During this time the "storage lesion" develops, characterized by changes in cation gradients, metabolism, oxidation, and vesiculation. Although there has been a lot of research into the different elements of the storage lesion, it is not yet clear in what order these defects occur and the sequence of cause and effect (Hess, 2010). Furthermore, very little attention has been given to the cation leak and how it contributes to, or maybe even triggers, other elements of the storage lesion. In this paper we will review the literature on RBC storage. We will consider the time-line of storage, the cause and effect of the different elements of the storage lesion, and we will describe certain variations that occur in the properties of donor blood. Other aspects of RBC storage are discussed in two companion papers of this research topic "Regulation of red cell life-span, erythropoiesis, senescence and clearance" (Bosman, 2013; Lutz and Bogdanova, 2013).

DONOR RBCs ARE A MIXED POPULATION

Donor RBCs are a mixed population of cells at the time of donation, ranging from newly formed reticulocytes through to 120 day old RBCs that are about to be removed from circulation. There is some evidence to suggest that during storage RBCs are in a state of suspended animation and do not continue to "age" as they would in the circulation at body temperature. The protein 4.1a/b ratio, an indicator of RBC age, doesn't change through storage (Minetti

et al., 2001). A recent study on reticulocyte maturation, by our group in Bristol, also provided evidence that reticulocytes do not mature in storage. In this study it was shown that reticulocyte maturation involves the formation of endocytic vesicles which then merge with autophagic vesicles forming large GPA and LC3 (autophagy marker) positive vesicles containing mitochondria etc. (Griffiths et al., 2012). These large internal GPA/LC3 positive vesicles can be seen in a small number of RBCs throughout the 35 days storage period suggesting that reticulocytes in donor blood at the time of donation do not mature significantly during the storage period. So at any given moment during RBC storage the unit will still contain a mixed population of cells from reticulocytes through to pre-senescent RBCs. Whether these RBCs, at different stages of maturity, succumb to the storage lesion equally has not been established but there are some indications that the lesion may affect young, middle and aged stored RBCs differently (Snyder et al., 1985).

METABOLIC CHANGES

The metabolic changes that occur in RBCs during storage have been extensively studied (Brewer et al., 1976; Strauss et al., 1980; Messana et al., 2000; reviewed in Hess and Greenwalt, 2002; Kanias and Acker, 2010; Buehler et al., 2011; Hess, 2014). RBCs lack mitochondria and are completely dependent on glycolysis for their energy requirements. RBCs are stored at 4°C, a temperature that slows metabolism, reducing the production of ATP and any RBC functions that require energy. The rate of glycolysis depends on temperature but more importantly on pH. The pH also affects the 2,3-diphosphoglycerate (2,3-DPG) level which in turn affects the oxygen carrying capacity of hemoglobin (Hb). Consequently, preservation of 2,3-DPG and adenosine triphosphate (ATP) levels by improved RBC storage solutions has been the focus of much research (Ambrus et al., 1975; Peck et al., 1981; Högman et al., 2006; reviewed in Hess and Greenwalt, 2002). A recent study reported in detail on the time-course of changes in metabolites during RBC storage (D'Alessandro et al., 2012). In summary their findings show that concentrations of metabolites such as fructose 1,6-diphosphate, glyceraldehyde-3-phosphate, total diphosphoglycerate, nicotinamide adenine dinucleotide (NAD^+) and ATP, all involved in the glycolytic pathway, increase during the first 7 days of storage, suggesting that at this early stage of storage glycolysis is proceeding. This increase in metabolites during the first 7 days of storage may in part be caused by the formation of deoxyhemoglobin which binds competitively to the N-terminal domain of band 3 (SLC4A1), displacing glycolytic enzymes and thereby activating them (Low et al., 1993; Messana et al., 2000). However, after day 7 the concentrations of these metabolites fall and that of phosphoenolpyruvate increases suggesting that glycolysis slows down. This is probably caused by the drop in pH, as lactic acid builds up, inhibiting glycolysis via a negative feedback mechanism. Lactate is excreted by the RBC *in vivo* and processed by the liver, however during blood storage lactate inevitably builds up in the bag.

As levels of the glycolytic metabolites diminish, the concentration of 6-phosphogluconate increases, as does nicotinamide adenine dinucleotide phosphate (NADPH) indicating that glycolysis is diverted down the pentose phosphate pathway (**Figure 1**). The pentose phosphate pathway produces NADPH which in turn reduces oxidized glutathione (GSSG), forming reduced glutathione (GSH) necessary for reduction of reactive oxygen species

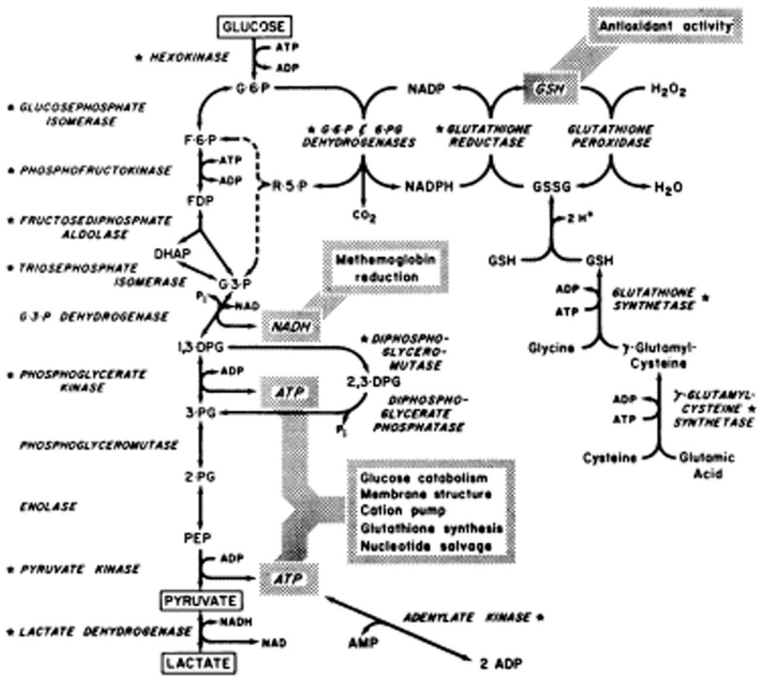

FIGURE 1 | Reprinted from Valentine (1979), with permission from Elsevier.

(ROS, **Figure 1**). Despite the increase of NADPH over storage there is not enough produced to maintain adequate levels of reduced glutathione; GSH falls continuously throughout the storage period and GSSG increases after day 14 (D'Alessandro et al., 2012).

EFFECT ON FUNCTION

The metabolic changes in the stored RBC affect the function of RBCs. The build up of lactic acid and fall in pH activates the phosphatase activity of diphosphoglycerate mutase, the enzyme that dephosphorylates 2,3-DPG (**Figure 1**). Hence levels of 2,3-DPG decline rapidly over the first week of storage (Bennett-Guerrero et al., 2007). Molecules of 2,3-DPG modulate oxygen transport by preferentially binding to deoxyhemoglobin and thus facilitate the release of oxygen in the tissues. Loss of 2,3-DPG causes the oxygen dissociation curve of stored RBCs to shift to the left (Hamasaki and Yamamoto, 2000; Opdahl et al., 2011). Molecules of 2,3-DPG also modulate membrane stability and thus deformation properties of RBCs by interacting with band 3 (SLC4A1) and protein 4.1 (EPB41) and disrupting the link between the membrane and the cytoskeleton (Moriyama et al., 1993; Chang and Low, 2001). Binding of 2,3,-DPG to N-terminal band 3 also affects the binding of glycolytic enzymes to band 3 modulating their regulation (Rogers et al., 2013). However, 2,3-DPG is thought to be replenished post-transfusion, although this may take >24 h (Hamasaki and Yamamoto, 2000), and so the oxygen carrying ability of hemoglobin in stored RBCs recovers eventually *in vivo*. Similarly ATP is probably replenished post-transfusion when lactate can be catabolized by the liver, relieving the pH stress and restoring glycolysis. ATP can certainly be restored *in vitro* by rejuvenating with the addition of certain metabolites and warming the RBCs. However, although the metabolic parameters can be improved by rejuvenation, the remaining elements of the storage lesion are more difficult to reverse (Tchir et al., 2013).

OXIDATION

The effect of oxidative stress on RBC aging is reviewed in detail in a companion paper of this research topic "Regulation of red cell life-span, erythropoiesis, senescence and clearance" (Mohanty et al., 2014). Here we will concentrate on the effect of oxidative stress on donor RBCs in storage.

Oxidative stress damages RBCs and shortens their life span (Fibach and Rachmilewitz, 2008). Reduced glutathione (GSH) is an important anti-oxidant molecule that "mops up" ROS (**Figure 1**). It has been shown that the amount of GSH present in RBCs decreases after day 14 of storage, while oxidized glutathione (GSSG) increases (D'Alessandro et al., 2012). The consequence is that oxidative damage increases, and this is reflected by an increase in malondialdehyde (MDA, a marker of lipid peroxidation) and protein carbonylation. Carbonylation, a marker of protein oxidative stress, increases from day 0 to day 28 (D'Alessandro et al., 2012) and occurs mainly on membrane and cytoskeleton proteins (Kriebardis et al., 2007a; Delobel et al., 2012). Carbonylation occurs earlier and more severely in CPDA-stored than CPD-SAGM-stored RBCs probably due to increased oxidative stress in CPDA-stored RBCs (Antonelou et al., 2010). Carbonylation of RBC protein decreases after day 28 perhaps because the oxidized protein is released in vesicles (D'Alessandro et al., 2012).

Oxidative stress may also be aggravated later in storage by iron release. Hemolysis increases over storage, releasing iron, which exacerbates the situation by causing oxidative damage and further hemolysis (Collard et al., 2013). Oxidative stress may also be increased in RBCs from glucose 6-phosphate dehydrogenase (G6PD) deficient donors. These donors provide 0.3% of RBC units in New York and a high proportion of them are R_oR_o phenotype (12.3% of the G6PD-deficient units in New York). This has implications for sickle cell patients; R_oR_o units are used preferentially for sickle cell patients who may be adversely affected by oxidized RBCs (Francis et al., 2013).

Alterations in cytoskeletal proteins (spectrin, protein 4.1, protein 4.2, dematin, ankyrin) are a clear indicator of oxidative stress. Other indicators are the recruitment of certain proteins to the membrane, for example the stress-response protein HSP-70, which then interacts with damaged cytoskeletal proteins (Antonelou et al., 2010). Anti-oxidant enzymes such as peroxiredoxin-2 (PRDX2, previously known as calpromotin) are also recruited to the membrane during oxidative stress (Antonelou et al., 2010; D'Alessandro et al., 2012) and associate with band 3 (Matte et al., 2013). Association of PRDX2 with the membrane in sickle cells is associated with activation of the calcium-activated potassium channel (Gardos channel), dehydration and dense cell formation (Moore et al., 1997). The oxidation of hemoglobin results in the formation of hemichromes which then bind to the RBC membrane, particularly to the N-terminal domain of band 3 (Kannan et al., 1988). Oxidation of the cytoskeleton and/or band 3 is thought to disrupt the cytoskeleton (permitting greater mobility of band 3), promoting the aggregation of band 3, which can lead to antibody binding (Pantaleo et al., 2009). Indeed, association of hemichromes with band 3 causes band 3 aggregation, increased immunoglobulin attachment and erythrophagocytosis in beta-thalassemia intermedia RBCs (Cappellini et al., 1999). Oxidative damage also occurs in other membrane proteins [e.g., glyceraldehyde-3-phosphate dehydrogenase (G3PD)] inducing proteolysis, protein aggregation and cross-linking. The effects of oxidation are ameliorated when oxygen is excluded (D'Amici et al., 2007).

VESICULATION

Vesiculation in both normal and hereditary hemolytic anemia RBCs is reviewed in detail in a companion paper of this research topic "Regulation of red cell life-span, erythropoiesis, senescence, and clearance" (Alaarg et al., 2013). Here we will concentrate on vesiculation of donor RBCs in storage.

Vesiculation during RBC storage has two important effects. Firstly the vesicles build up in the blood bag and are transfused into the patient, and secondly vesiculation causes irreversible damage to the RBCs because once membrane has been lost the RBC cannot regain its original morphology. Vesicles produced by cells can be divided into three main groups; exosomes (30–100 nm), microvesicles or ectosomes (0.1–1 μm), and apoptotic bodies (1–5 μm) (Gyorgy et al., 2011). What is less clear is the sidedness of each of these types of vesicle. This has some clinical significance because if the vesicles produced in storage

are right-side out then they are likely to be maintained in the circulation for much longer. Vesiculation may be used to rid the cell of unwanted or damaged proteins/components, in which case the vesicles need to carry a signal for immediate disposal. Alternatively, the vesicles may be used to communicate between cells, in which case they need to persist in the circulation at least until they have delivered their message.

Vesicles formed in order to carry messages between cells would need to be right-side out as immediate removal by macrophages would be counter-productive. Right-side out vesicles can be formed by simple blebbing or budding of the plasma membrane (ectosomes/microvesicles) or by the production of exosomes. Exosomes are formed by initial endocytosis producing an inside-out internal vesicle which then invaginates to form a multi-vesicular body (MVB) containing right-side out exosomes. Although immature RBCs (reticulocytes) are thought to produce exosomes via formation of MVBs (Blanc and Vidal, 2010), mature RBCs lack this capacity and release only microvesicles/ectosomes (Alaarg et al., 2013). RBCs shed right-side out vesicles *in vivo* (Dumaswala and Greenwalt, 1984), and these may communicate with other intact cells. Indeed, vesicular transfer of glycosylphosphatidylinositol-linked proteins has been shown to occur, correcting the defect in paroxysmal nocturnal hemoglobinuria (PNH) RBCs (Sloand et al., 1998, 2004). These right-side out vesicles are also implicated in inflammatory and immunomodulatory responses to transfusion (Sadallah et al., 2008, 2011; Kriebardis et al., 2012).

Vesiculation may be used to remove damaged proteins from the mature RBC membrane and prolong the life of RBCs *in vivo* (Bosman et al., 2012). There is evidence that vesiculation to remove damaged protein also occurs in stored RBCs. Electron and confocal microscopy images show that during storage the morphology of RBCs changes from discocyte to echinocyte, and that vesicles appear to form at the tips of the echinocytic spicules (Tissot et al., 2010), suggesting that vesicles produced in stored RBCs are ectosomes. Ectosomes would be expected to form in a right-side out manner, which would not expose phosphatidylserine (PS; a phospholipid located exclusively on the inner leaflet of the membrane bilayer). However, studies have found that about a third of storage-induced RBC ectosomes do expose PS on their surface (Salzer et al., 2008). Ectosomes of platelet origin have also been reported to be a mixture of PS positive (20%) and PS negative (80%) vesicles (Connor et al., 2010). So the cell must have some mechanism for exposing PS at the tips of the spicules. It is possible that damaged protein destabilizes the packing of the transmembrane proteins and the cells integrity, allowing localized entry of calcium. Lipid rafts may form around the damaged protein, calcium may activate scramblases and translocation of PS to the outer leaflet and result in the formation of a PS-exposing bleb.

Indeed there is some evidence that spiculation and vesiculation may be dependent on PS exposure. In Scott syndrome, a bleeding disorder, PS exposure is inhibited in both platelets and RBCs. Loss of PS exposure in these RBCs (and platelets) impairs the formation of echinocytes and calcium-induced vesiculation (**Figure 2**; Bevers et al., 1992). The Scott syndrome patient was found to have a splice site mutation in *TMEM16F*, now known as anoctamin 6 (*ANO6*) (Suzuki et al., 2010). This protein has since been found to form a calcium-activated cation channel required for lipid scrambling (Yang et al., 2012).

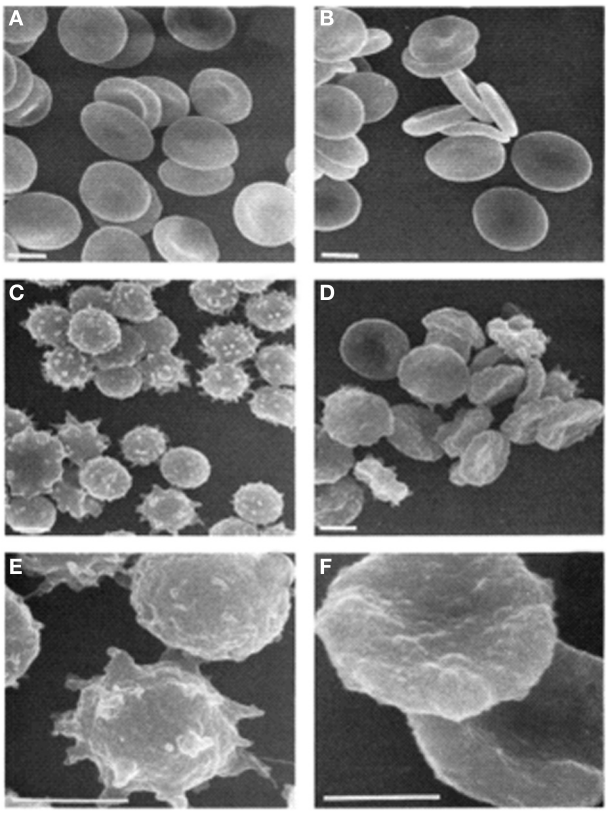

FIGURE 2 | Scanning electron micrographs of RBCs. (A) Untreated control; **(B)** untreated Scott syndrome; **(C,E)** A23187-treated control; **(D,F)** A23187-treated Scott syndrome. Bar indicates 3 μm. Magnification in **(E,F)** is three times higher than in **(C)** through **(D)**. Cells were fixed 1 h after addition of ionophore. Reprinted from Bevers et al. (1992), with permission from Elsevier.

It is worth noting that there are differences between storage-induced RBC vesicles and calcium-induced RBC vesicles, which are produced by incubating RBCs with a calcium ionophore in the presence of calcium. Both types of vesicle contain detergent-resistant material (DRM) comprising of lipid rafts and are rich in the raft proteins acetylcholinesterase, CD55, flotillins, stomatin (Salzer et al., 2008), and are free from cytoskeleton proteins (De Jong et al., 1996), although actin is present (Kriebardis et al., 2008). Storage-induced vesicles are a similar size to calcium-dependent vesicles, but contain more stomatin and less flotillin (Salzer et al., 2008). Calcium ionophore treatment was shown to produce vesicles rich in stomatin, synexin (annexin VII), and sorcin. The latter two proteins are usually cytosolic but are recruited to the membrane upon stimulation with calcium (Salzer et al., 2002). Synexin and sorcin have been reported to be recruited to the membrane in stored RBCs, leading the authors to suggest that calcium-dependent processes are likely to occur in RBCs during storage, despite the presence of citrate in the storage solution, which chelates calcium (Kriebardis et al., 2007b).

Band 3 is an important protein for the structure and morphology of the red cell because it links the membrane to the underlying spectrin cytoskeleton. This is achieved via a strong association with the linker protein ankyrin. Studies suggest that phosphorylation of band 3 disrupts its interaction with ankyrin and therefore weakens the membrane-cytoskeleton link (Ferru et al., 2011; Pantaleo et al., 2011). A high state of phosphorylation on band 3 was shown to result in echinocytic morphology and increased microvesicle release (Ferru et al., 2011), providing another mechanism for vesiculation.

Band 3 and Hb are found in vesicles throughout storage, probably representing the non-attached band 3 (Bosman et al., 2008). The amount of CD47, a marker of self, increases in vesicles during storage and this loss of CD47 from stored RBCs may affect RBC clearance post-transfusion (Anniss and Sparrow, 2002). Some proteins, for example complement receptor 1 (CR1), are enriched in vesicles (Pascual et al., 1993) but other proteins are absent from vesicles, for example aquaporin 1, suggesting that there is sorting of proteins into vesicles while others are preferentially retained (Kriebardis et al., 2008). By day 35 of storage vesicle formation proteins such as alpha SNAP can be found at the membrane (D'Alessandro et al., 2012). Vesicles also carry a large amount of immunoglobulins. Fas-related signaling molecules such as Fas-associated death domain (FADD) and caspase 8 are present in vesicles and the amount of vesicle-associated Fas and caspase 3 increases with storage time. Fas is thought to segregate to lipid raft domains, activate caspases which inhibit aminophospholipid translocase (flippase) activity and cause PS exposure (Mandal et al., 2005). As previously mentioned, about one third of vesicles expose PS (Salzer et al., 2008), and transfusion of PS-exposing vesicles may have pro-coagulatory effects leading to thrombo-embolic complications (Owens and Mackman, 2011; Rubin et al., 2012). In some situations this may be of benefit to stop bleeding (Jy et al., 2011; Kriebardis et al., 2012). PS-exposing vesicles may also recombine with other cells, labeling their surface with PS and marking them for destruction. Vesicles also carry Hb, which can increase the iron-load of the transfused patient, and can deplete nitric oxide, inhibiting vasodilation.

CATION GRADIENT DISSIPATION

All RBCs (circulating or stored) have a slight permeability to monovalent cations. The RBC membrane provides a permeability barrier, enabling the cell to maintain different concentrations of ions internally, but this barrier is not perfect and ions leak down their concentration gradients. This minor leak is constantly corrected by the NaKATPase which pumps potassium into the cell in exchange for sodium and maintains a gradient such that in humans RBCs contain ∼90 mM potassium and ∼5 mM sodium whilst the plasma contains ∼5 mM potassium and ∼140 mM sodium. However, donated RBCs are stored at 4°C, and at this temperature the NaKATPase has limited functionality (Marjanovic and Willis, 1992), even before ATP becomes limiting, so cations leak across the RBC membrane unopposed until they find an equilibrium with the external medium (Wallas, 1979). As discussed above, metabolic and oxidative changes develop throughout the storage period, but the cation leak is apparent immediately and may be responsible for early changes in stored RBCs; in membrane potential, cell volume and morphology (Berezina et al., 2002). After 35 days storage the extracellular solution of a leukodepleted unit of packed RBCs contains ∼40–50 mM potassium (Bawazir et al., in press). Concurrently the intracellular concentrations of monovalent cations change in stored RBC; potassium concentration is reduced and intracellular sodium levels increase, although this is rarely reported. The consensus is that cation gradients can be restored in RBCs post-transfusion. Restoration of cation gradients, after incubation with glucose at 37°C, was reported many years ago (Flynn and Maizels, 1949) however this study used 6-day stored RBCs, when ATP levels would be high. Conversely, a more recent study showed that overnight incubation at 37°C of 35-day stored RBCs increased potassium leakage, hemolysis, PS exposure, and vesiculation (Burger et al., 2013).

The altered cation gradient across the RBC membrane of RBCs stored in SAG-M has a distinct effect on cell volume and shape. This can be seen by comparing circulating RBCs with stored RBCs. Both types of cell develop oxidized protein and lipid and vesiculate, however cell volume changes differ in the two cell types. In circulating RBCs the cells start off large and become small, in stored RBCs the MCV becomes larger throughout storage. This difference may in part be due to the presence or absence of calcium. The dehydration of old circulating RBCs is thought to involve the calcium-activated Gardos channel whereas the storage solution of stored RBCs contains some citrate, which chelates calcium. The different volume changes in circulating and stored RBCs suggests the mechanism of vesiculation may be different in the two cells types. In circulating RBCs the cation gradient is maintained and the shape, volume and cytoskeleton integrity are maintained until the cell becomes damaged. Then controlled vesiculation occurs in order to remove damaged protein (Bosman et al., 2012). About 20% of the RBC membrane may be lost in this way during the 120 day lifetime of a RBC. Eventually small, dense, spherocytes result and are removed from the circulation by phagocytosis. In stored RBCs the cation gradient is lost gradually and the cell shape and volume are altered. The cells swell, weakening the cytoskeleton which is also weakened by oxidation and ATP loss. The membrane becomes unstable, echinocytes form and membrane is lost by vesiculation, seemingly a much less controlled mechanism (**Figure 3**). Even at day 5 of storage the membrane appears less tightly controlled with some swollen, misshapen discocytes present (**Figure 3A**).

By day 21 of storage the osmotic fragility of the RBCs is increased and more than 50% of the cells display non-discocyte morphology (Blasi et al., 2012). By day 35 of storage the morphology of about 25% of RBCs is irreversibly altered; the cells have lost membrane through vesiculation and become spherocytic (spheroechinocytes, spherostomatocytes, spherocytes) (Blasi et al., 2012). About 25% of long-stored RBCs are immediately removed from the circulation post-transfusion (Luten et al., 2008). It is likely that the irreversibly-altered, echinocytic spherocytes in long-stored RBCs form this fast-removed population (Beutler et al., 1982). It may not simply be PS expression that triggers their removal, indeed PS exposure on stored RBCs remains quite low (Verhoeven et al., 2006), only beginning to increase around day 28 of storage (Dinkla et al., 2013). It may also be

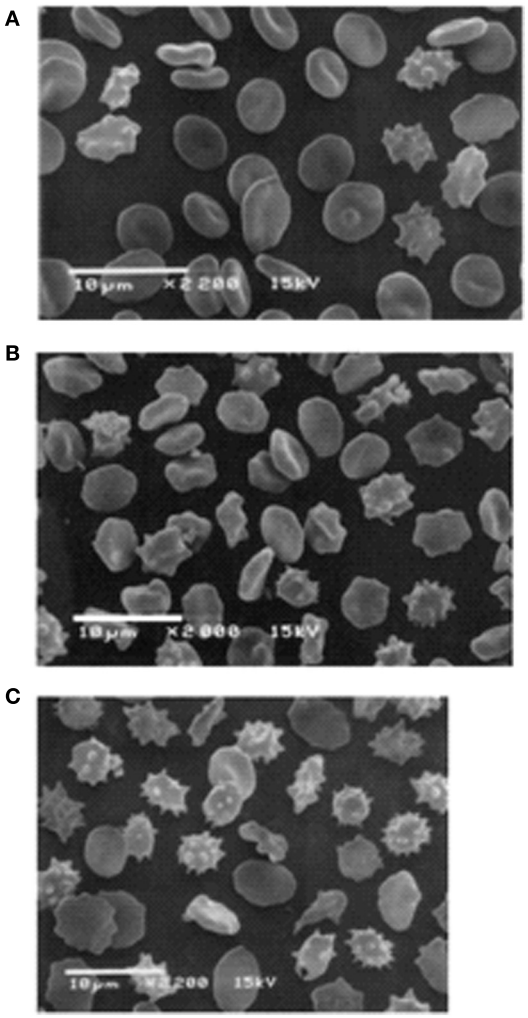

FIGURE 3 | Scanning electron microscope pictures of RBC. Stored RBC on the 5th **(A)**, 14th **(B)**, and 42nd **(C)** day of storage. **(A)** Discocytes dominate among the cell population, and only a few irreversibly changed RBC can be seen. **(B)** Numerous echinocytes and spheroechinocytes can be seen. **(C)** Spheroechinocytes and degenerated forms dominate among irreversibly changed cells. Cells stored in adenine saline solution (AS-3 contains saline, adenine, glucose, phosphate, and citrate). Reprinted from Berezina et al. (2002), with permission from Elsevier.

due to the rigidity of these cells, which would resemble that of hereditary spherocytosis cells.

THE ORDER OF THE STORAGE LESION

Many studies have tried to unravel the order in which the different elements of the storage lesion occur. The current accepted theory holds that during storage ATP levels fall, ROS increases and the RBC membrane becomes oxidized. This causes disruption of the cytoskeleton, aggregation of band 3 and release of vesicles. There is significant evidence supporting this course of events. ATP levels, after rising during the first week of storage, fall away from day 7 onwards (D'Alessandro et al., 2012). ROS levels increase gradually for the first week of storage and then rapidly increase to a maximum by the second week of storage (D'Alessandro et al., 2012). Oxidation is shown to reduce the spectrin–actin interactions during storage (Wolfe et al., 1986) and correlates with vesicle release (Wagner et al., 1987). Aggregation of the mobile pool of band 3 coincides with increased ROS and oxidation of the RBCs (Kriebardis et al., 2007a; Karon et al., 2012; Arashiki et al., 2013) and occurs before vesiculation (Karon et al., 2009). Deformability of stored RBCs decreases throughout storage as cells lose membrane and become spherical (Bennett-Guerrero et al., 2007). Consequently much effort has been put into maintaining ATP levels in stored RBCs.

ASSOCIATION OF MCV AND RDW WITH THE CATION LEAK

However, this sequence of events ignores the impact of the cation leak which occurs from day 1 of storage. It is possible that the change in cation distribution initiates some of the other components of the storage lesion. The cation leak in stored RBCs causes a redistribution of monovalent cations but also an overall uptake of base (and therefore water) which causes the cells to swell (Flynn and Maizels, 1949). In circulating RBCs loss of cation gradients and cell swelling may activate the Gardos channel, resulting in loss of potassium (and cell shrinkage), but this mechanism requires calcium which is reduced in stored RBCs due to the presence of citrate in the CPD-SAGM. So the overall effect of cation changes is for there to be an increase in mean cell volume (MCV). Indeed the MCV of RBCs stored in SAG-M increases steadily from day 1 and throughout storage (Antonelou et al., 2012; Bawazir et al., in press). However, during storage some cells lose membrane by vesiculation and become smaller and spherocytic. Therefore, the remaining cells must be even more swollen for the MCV to continue to rise, and this is reflected in the steady increase in red cell distribution width. **Figure 3** shows the variation in size and shape of red cells stored in AS-3. By day 35 of storage (**Figure 3C**) the cells range from large and misshapen to small, spherocytic echinocytes. Even at day 5 of storage (**Figure 3A**) both these cell types can be seen and this is before any ATP loss or a significant rise in ROS. ATP levels are known to rise over the first 7 days of storage, and ROS and protein carbonylation increase only slowly for the first 7 days, then rapidly between day 7 and day 21, when they reach a plateau (D'Alessandro et al., 2012; Suppl Figure S2). So, although ROS may contribute to these early morphological changes in the RBCs, changes in cation gradients probably play an important role.

It should be noted here that this increase in MCV does not always occur when RBCs are stored in other storage media. Although all stored RBCs leak cations, regardless of the storage medium, different storage media affect MCV and osmotic fragility in different ways (Zehnder et al., 2008; Veale et al., 2011). The MCV of RBCs stored in PAGGSM remains fairly constant throughout storage and the MCV of RBCs stored in Erythrosol-4 decreases throughout storage (Veale et al., 2011). These storage media related differences were explained by differences in vesiculation (Veale et al., 2011), although another study found no difference in vesiculation (Zehnder et al., 2008) and vesiculation can vary enormously between different donors (Rubin et al., 2008; Lion et al., 2010). These storage media related differences cannot be explained by differences in Gardos channel activity. Although the MCV of RBCs stored in Erythrosol-4 decreased

steadily through storage, Erythrosol-4 contains 25 mM Na-citrate (Veale et al., 2011).

ASSOCIATION OF PS EXPOSURE WITH THE CATION LEAK

The impairment of PS exposure in Scott syndrome, discussed above, suggests PS exposure may be a prerequisite for the formation of echinocytes and RBC vesiculation. It has been shown that the high intracellular potassium concentration of fresh RBCs inhibits lipid scrambling activity (Wolfs et al., 2009). So it follows that when the stored RBC leaks cations and the intracellular potassium levels go down, scrambling may increase and more PS may be exposed. At the same time the cation leak causes overhydration of the stored RBCs in SAG-M (as discussed above) resulting in large misshapen cells. Together these two effects of the cation leak, overhydration and PS exposure, may initiate the early changes in RBC morphology creating protrusions or spicules on the cell membrane. One study appears to support this hypothesis. The authors found that reduced intracellular potassium caused decreased flippase activity, causing PS exposure and vesiculation (Burger et al., 2013). This was an *in vitro* study that used an overnight incubation at 37°C to mimic post-transfusion conditions. It showed that flippase activity was reduced under these conditions, however the study did not report the ATP levels of the stored RBCs and lack of ATP may have contributed to these findings. In another study it was reported that scramblase activity is virtually absent during RBC storage, and flippase activity, although reduced after 21 days storage due to lack of ATP, can be restored if metabolic changes are corrected (Verhoeven et al., 2006).

ASSOCIATION OF VESICULATION WITH THE CATION LEAK

Vesiculation increases with prolonged storage but increases markedly after day 21 (Rubin et al., 2008; Lion et al., 2010). Vesiculation seems to occur partly as a result of the cation leak causing cell swelling and PS exposure (as discussed above), and partly by the oxidation of the membrane in particular the cytoskeleton. Certainly oxidation of cytoskeleton/spectrin has been shown to lead to vesiculation. Prolonged storage weakens the spectrin–actin-protein 4.1 interactions and this effect is probably due to oxidation of spectrin or other cytoskeletal proteins and can be reversed in part by treatment with a reducing agent, dithiothreitol (Wolfe et al., 1986). It has also been shown that there is variation in the number of vesicles produced from RBCs stored in different storage media; those media that efficiently combat oxidative stress produce fewer vesicles (Dumaswala et al., 1996; Antonelou et al., 2010; Veale et al., 2011). Similarly, another study has shown that the expression of aging markers such as aggregation and proteolysis of band 3 and increased binding of hemoglobin and autologous antibodies are more pronounced in CPDA than CPD-SAGM suggesting that oxidation is involved (Antonelou et al., 2010). So although the cation leak may initiate changes in the membrane that lead to vesiculation in the early stages of storage, oxidation of the membrane doubtless plays a significant role in vesiculation after the second week of storage.

ASSOCIATION OF OXIDATION WITH THE CATION LEAK

Interestingly, oxidation of stored RBCs and the effects of the cation leak in stored RBCs appear to be intertwined. Some red cell concentrate units are subjected to doses of gamma irradiation in order to prevent graft-vs.-host disease in vulnerable patient groups. This process is associated with increased oxidative damage to the red cell membrane as well as an increased potassium leak (Serrano et al., 2013). It has been shown that oxidation of the cytoskeleton may exacerbate the cation leak by weakening the integrity of the membrane (Deuticke et al., 1984; Ney et al., 1990). Lipids are also oxidized and lipid hydroperoxides may permit a deformation-dependent leak of monovalent cation from erythrocytes (Sugihara et al., 1991) although this is disputed by others (Deuticke et al., 1987). Indeed the oxidation and deformation effects on the RBC cation leak are synergistic (Ney et al., 1990). Equally, the cation leak may also exacerbate oxidation of the stored RBCs. Long-term storage of human RBCs is associated with a decrease in the concentration of glutathione because of a reduced rate of synthesis (Whillier et al., 2011). This was found to be caused by both decreased ATP concentration and reduced amino acid transport (Whillier et al., 2011). As certain amino acid transporters are sodium dependent, such as SLC1A5 the Na-dependent neutral amino acid transporter that transports glutamine and asparagine, the reduction in amino acid transport could in part result from loss of sodium gradient across the membrane.

The above discussion by no means resolves the order in which the different elements of the storage lesion occur but may go some way to highlight how all of these factors are interlinked. Hopefully it also makes a case for more attention to be paid to the cation leak and its effect on the other components of the storage lesion. Reduction of the cation leak, perhaps by improvements in storage media, may delay or ameliorate other components of the lesion. Something that has become clear recently is that there is wide variation in severity of the storage lesion amongst blood donors, in particular variation in cation leak.

VARIATIONS IN DONOR RED CELL PROPERTIES

Individual blood donors can show a wide variation in the properties of their red cells. This can be as a result of lifestyle and diet. Indeed, it has already been discussed in this series of articles that some athletes' circulating red cells represent a younger population because of intravascular hemolysis during impact sports (Mairbäurl, 2013). At the other end of the spectrum, it is well established that an increase in erythrocyte MCV often accompanies chronic alcoholism (Wu et al., 1974). It has also been observed that there is a large variation in the number of RBC vesicles shed by different donors, but the reason for this is unknown (Rubin et al., 2008; Lion et al., 2010). Some blood parameters vary according to age and gender; but in addition to this, variation in donated cells can have a genetic basis. There is evidence from genome-wide association studies that single nucleotide polymorphisms (SNPs) in certain genes are linked to variations in the properties of donors' red cells, such as MCV and mean cell hemoglobin (MCH) among others (Ganesh et al., 2009). Indeed, approximately 25 percent of black blood donors have elevated RBC sodium (Na_i) level compared with white donors, probably due to an unknown genetic change. This elevation results in a significant increase in the mean Na_i from black

(9.00 ± 2.96 mmoles/L RBC) as compared to white blood donors (7.04 ± 1.48 mmoles/L RBC, $p < 0.001$) (Wallas et al., 1982).

We have shown that in the donor population there is a natural variation in the rate of the cation leak across the red cell membrane (Bawazir et al., in press). In everyday life this variation is of no consequence, because the correcting action of the NaKATPase easily maintains the cation gradients necessary for cellular volume control and hydration. Once the red cells are donated and stored, this natural variation becomes measureable as the action of the NaKATPase is ablated by cold-storage. At day 35 of storage most units have 40–45 mM extracellular potassium but some units have much higher levels (up to 80 mM), and some have much lower levels (10 mM) (**Figure 4**). The majority of variation in potassium levels in units after 35 days' storage is probably due to environmental factors, but there may be genetic markers that are associated with a particularly high or low rate leak.

FP-CARDIFF AND THE HIGH-RATE CATION LEAK

Consistent with the idea that some aspects of the red cell cation leak can have a genetic basis, we have identified a SNP in a red cell membrane protein that co-segregates with a high cation leak condition in a three-generation family. This mutation has so far been found in two unrelated UK blood donors (Bawazir et al., in press). This particular condition, called FP-Cardiff, forms part of a larger family of disorders that share the common feature of cation-leaky red cells. Collectively, they are known as the hereditary stomatocytoses (Stewart, 2004; Gallagher, 2013). Unlike the other members of the hereditary stomatocytoses FP-Cardiff has relevance to blood donation and storage because carriers of this mutation are not anemic, show no disease symptoms and therefore may become blood donors. However, once their blood is cooled for storage the red cell membrane exhibits an unusually high rate of cation leak such that potassium levels in the unit can be 40 mM by day 5 (Bawazir et al., in press). Ultimately, as with normal units, the levels of supernatant potassium in FP-Cardiff units will plateau once an equilibrium is reached between the intracellular and extracellular space.

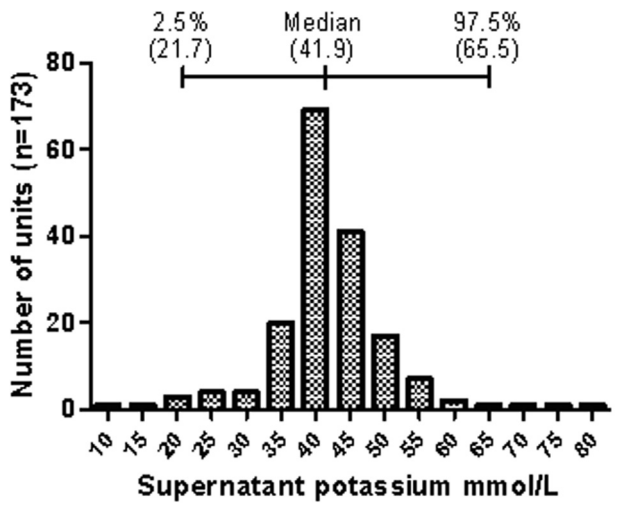

FIGURE 4 | Distribution of supernatant potassium concentration of stored donor red cell units. The graph shows the distribution of the potassium concentration in the supernatant of red cell units at day 35 of refrigerated storage as collected by the Components Development Laboratory, NHSBT. Data represents randomly selected units collected between 2003 and 2006. The median and median percentile data are shown above the bar chart. Reprinted from Bawazir et al. (in press), with permission.

Under normal circumstances the transfusion of high-potassium units is unlikely to affect the recipient adversely, because the transfusion of blood is slow and the relatively large volume of the recipient's own circulating blood will dilute the potassium to safe levels. However, in certain situations, such as large volume or exchange transfusions for neonates, there is a requirement for low-potassium units. In these cases it is normal practice to use blood that has been stored for less than 5 days in order to avoid high supernatant potassium. Unusually high levels of potassium may be present in short-stored blood packs if they have come from a donor with FP-Cardiff (or other condition that results in a high rate of potassium leak during blood storage). The consequences of a high potassium transfusion can be serious, including cardiac arrest (Vraets et al., 2011). Interrogation of the available databases suggests that FP-Cardiff could be present in 1 in 500 people in the European population (Bawazir et al., in press).

Aside from the risk of transfusion-associated hyperkalemia, it is not yet known if the accelerated loss of the cation gradients in high-leak red cells speeds up or exacerbates the other features of the storage lesion. As the correct hydration of red cells is so dependent on these cation gradients one might expect that the changes in MCV and red cell morphology that are observed over storage may be accelerated in FP-Cardiff units. An increase in the formation of microvesicles and/or sphero-echinocytic cells would be a concern because of the potential inflammatory and thrombotic effects of microvesicles, and the fact that the spleen will immediately remove the majority of spherocytic cells (Safeukui et al., 2012). Further work to characterize FP-Cardiff red cells and establish how they respond to prolonged storage is currently ongoing in our laboratory. The study of these cells will be a useful tool to investigate the relationship of the cation leak with vesiculation, membrane oxidation and other hallmarks of the storage lesion.

CONCLUDING REMARKS

There is still some debate among clinicians over whether the duration of blood storage really matters (Cheuk, 2012; Wang et al., 2012). It is, however, undeniable that certain changes do occur once the blood donation is given, the pack is processed and the cells are kept in cold storage for as long as 42 days. A large amount of work has been done to characterize the red cell storage lesion, and we are now beginning to understand the order in which the various elements occur during storage. One of the problems associated with older blood is the loss of cation gradients across the red cell membrane. This aspect has hitherto not received the degree of attention afforded to other lesion hallmarks such as vesiculation and oxidative damage. It appears to be the case that most elements of the lesion, if not all of them, show some degree of inter-dependence. As such, the contribution of the cation leak is beginning to be better understood but more

work will be necessary to untangle the intricacies of these complex relationships. Further studies to understand the cause and effect of the different elements of the lesion are key to ameliorating the initiating factor(s) and reducing the lesion.

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript preparation.

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