Chapter

Erythrocytes as Biomarkers of Virus and Bacteria in View of Metal Ion Homeostasis

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Abstract

The erythrocyte contributes to the immune system in several ways. It sequesters interferons, interleukins or chemokines and by binding nucleic acid. It binds virus and bacteria and may deliver bacteria to macrophages for phagocytosis. It may also kill bacteria directly with oxygen. For proper function of the erythrocyte, homeostasis of reactive oxygen species, selenium, metal ions and trace elements is important. Erythrocytes display morphological and metabolic changes in diseases like sepsis, and in several genetic diseases. Patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), giving rise to the coronavirus disease 2019 (Covid-19), show many erythrocyte changes as compared to healthy controls. The erythrocyte responds to hemolysins by purinergic signaling leading to hemolysis or phosphatidylserine exposure on the plasma membrane. Phosphatidylserine marks erythrocytes for clearance by spleen macrophages. Regulated erythrocyte cell death, also called eryptosis, can be induced by oxidative stress, pathogen infection, and certain diseases like sepsis. Erythrocytes may, in the future, contribute more to diagnosis based on research and diagnostic technological development.

Keywords: Erythrocyte, cytokine, sepsis, reactive oxygen species, selenium, iron, calcium, metal ions, ICP-MS, hierachy, Covid-19

1. Introduction

Recent research has shown that the erythrocyte contributes in many ways to defense against pathogens. The human erythrocyte is infected by the unicellular eukaryotic parasites *Plasmodium* and *Babesia* and the bacterium *Bartonella* [1]. Bacteria may adhere to glycosylated erythrocyte membrane proteins thereby facilitating phagocytosis by macrophages. Bacteria adhering to the erythrocyte surface may also be killed by oxygen release [2]. The erythrocyte also reacts with morphological changes to infections like Covid-19 or a disease like sepsis. However, the mammalian erythrocyte, having lost its nucleus and organelles, seems to play less a role in the immune system than erythrocytes of fish and birds [3]. The chapter will review aspects of mainly human erythrocytes in immunology and homeostasis with some special attention given to the ongoing Covid-19 pandemic.

2. The erythrocyte in immunology

Erythrocyte surface antigens play an important role in the erythrocyte interaction with pathogens and in other immune reactions. To begin with, the Duffy antigen has been found to be the entry point for *Plasmodium vivax*. This is supported by the finding that Duffy-negative individuals have some protection against *P. vivax* infection. Duffy negativity is common in the African population, although *P. vivax* may be able to infect some Duffy negative individuals [4]. Duffy was found to be a receptor of the chemokine with Cysteine-X-Cysteine (C-X-C) motif ligand 8 (CXCL8, also called interleukin-8) (**Figure 1**), and other chemokines such as chemokine with Cysteine-Cysteine (C-C) motif ligand 2 (CCL2, also called macrophage chemoattractant protein-1 (MCP-1)) were later also found to bind to Duffy [5]. The Duffy protein is a putative G-protein coupled receptor, but lacks some parts that would be necessary for signaling and is therefore regarded as a non-signaling receptor. The current hypothesis is that Duffy functions as a buffer-storage-sink for chemokines in the body. One indication for this is the reduced neutrophil counts and increased risk of acquiring human immunodeficiency virus (HIV-1) that has been found in Duffy-negative individuals [6]. However, overall neutrophil effector functions were largely unaffected in HIV-1-infected and non-infected Duffy-negative individuals [7]. One explanation for this could be an increased proteolytic activity as a compensatory mechanism for lower absolute neutrophil counts [7].

Chemokines belong to a group of immune signaling proteins called cytokines. In addition to chemokines, interferons, interleukins and the hormone erythropoietin are classified as cytokines. From the initial discovery of chemokine binding to Duffy, many more cytokines have been shown to be associated with the erythrocyte [8]. A considerable step forward was taken in a study where almost 50 different cytokines were identified in erythrocyte preparations [9]. Among chemokines, several from the C-C and the C-X-C families were found. Among interleukins, the interleukin-1, interleukin-6, interleukin-12 and interleukin-17 families were present (**Figure 1**). Present were also granulocyte-macrophage colony stimulating factor (GMCSF), interleukin-3, interleukin-5, tumor necrosis factor (TNF) superfamily and platelet derived growth factor (PDGF) (**Figure 1**). Interferon type I was represented by interferon-alpha2 whereas interferon type II was represented by interferon-gamma (**Figure 1**). The study suggests that the erythrocyte has significant capacity to contribute to the cytokine pool and immune homeostasis.

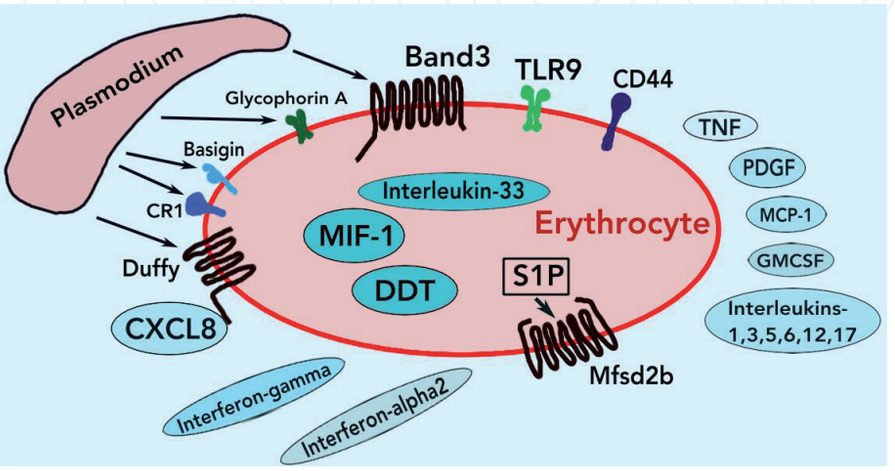

Figure 1.
Schematic figure of the erythrocyte in immunology. An erythrocyte contains 1.2 million band3 and 0.5 million glycophorin a molecules. Abbreviations are explained in the text.

In addition, cytokine and cytokine receptor gene expression has been found in both human and murine erythroblasts [10, 11].

Most of the cytokines have been localized extracellularly on the erythrocyte possibly bound to transmembrane receptors or associated to the glycocalyx. Macrophage migration inhibitory factor 1 (MIF1), D-dopachrome tautomerase (DDT) and interleukin-33 were found to have an intracellular localization [8, 12] (**Figure 1**). Interleukin-33 is a nuclear localized interleukin belonging to the interleukin-1 family [12]. Interleukin-33 is probably left over after expelling of the nucleus from the reticulocyte. MIF1 and DDT were found to exist in the erythrocyte, although the exact receptor has not been identified. In other tissues, a receptor for MIF1 and DDT is formed by CD74 and the hyaluronan receptor CD44 (**Figure 1**), which is also present on erythrocytes [13]. Intracellular as well as extracellular erythrocyte cytokines may be released by hemolysis or in protein-containing micro-vesicles or micro-particles produced by the erythrocyte [14–16].

Toll-like receptors (TLR) are a group of trans-membrane receptors of the innate immune system. TLR9 was shown to be present on erythrocytes [17] (**Figure 1**), although not all erythrocytes express TLR9 and considerable interindividual differences in expression was found [17]. Presence of mitochondrial but not nuclear DNA was confirmed on human and mouse erythrocytes. A knock-out mouse for TLR9 lost much of the mitochondrial DNA binding capacity otherwise found on human and mouse erythrocytes. TLR9 protein was mainly localized in the erythrocyte close to the plasma membrane probably binding to band3, a 14-transmembrane helices protein also known as anion exchanger 1 (AE1 or SLC4A1). Some evidence was found for erythrocyte scavenging of mitochondrial DNA from microvascular endothelial cells [17]. The erythrocyte may also interact with macrophages through the TLR9 receptor [18] (**Figure 1**). Additional TLRs may also exist in the erythrocyte, since expression of several TLRs was found in murine erythroblasts [11].

Erythrocytes also store and export the multifunctional molecule sphingosine-1-phosphate (S1P) (**Figure 1**). Inside the erythrocyte S1P has been shown to be involved in regulation of glycolysis [19]. S1P binds the S1P-receptor-1 on lymphocytes, antagonizing their egress from secondary lymphoid organs and the thymus. When bound to apolipoprotein M, S1P reduces lymphopoiesis by binding S1P-receptor-1 on common lymphoid progenitors in the bone marrow [20]. Erythrocytes synthesize S1P from sphingosine obtained from plasma in a reaction catalyzed by sphingosine-kinase-1. Because of lack of cellular organelles and their associated enzymes, no S1P-degrading enzyme is present in erythrocytes. These therefore effectively function as a S1P storage facility. Through site-directed mutagenesis and gene-knock-out studies, the major facilitator superfamily domain 2b (Mfsd2b) was found to be the exporter of S1P from erythrocytes [21]. Export of S1P from erythrocytes (**Figure 1**) was inhibited by an anion transport inhibitor, suggesting that also band3 may be involved in S1P export [22]. Erythrocytes are considered the major source of S1P plasma levels because of low plasma S1P levels in anemic patients. S1P plasma levels are also regulated by the lipid phosphate phosphatases, LPP1 and LPP3, on the plasma membrane of endothelial cells [23]. In addition, S1P levels in plasma may also depend on the presence and regulation of S1P carrier molecules like albumin and apolipoprotein M in complex with high-density lipoprotein.

Important for entry of *P. falciparum* into the erythrocyte are glycophorin A, band3, complement receptor 1 (CR1) and basigin [24] (**Figure 1**). Basigin is also known as the extracellular matrix metalloproteinase inducer (EMMPRIN) or CD147. The protein glycosylation underlying the ABO blood group system, is present on erythrocyte membrane proteins like band3. The ABO glycosylation is used by *Plasmodium* to facilitate rosetting, a stage in *Plasmodium* pathogenesis [25].

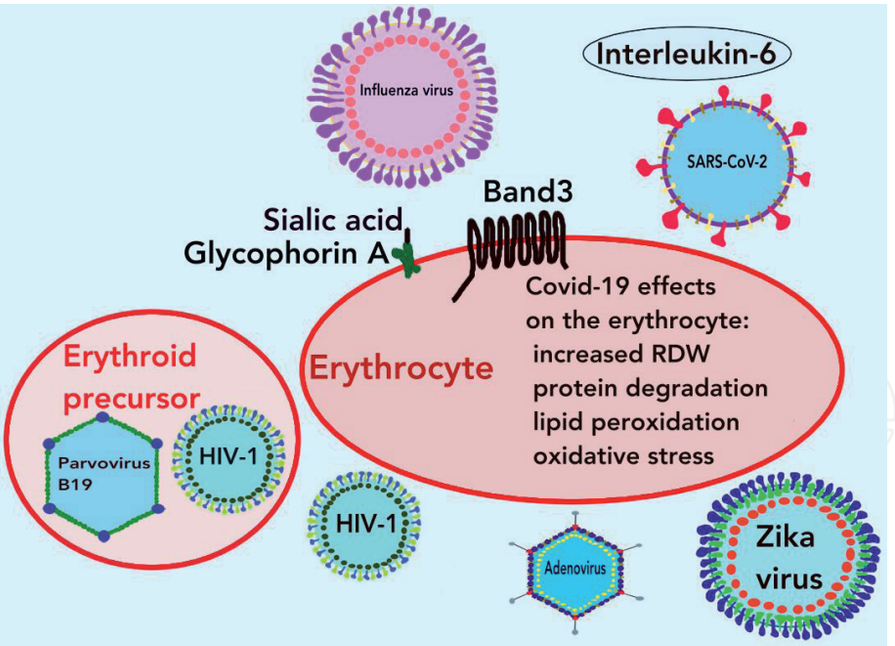

Figure 2.
Schematic figure of the erythrocyte and its interactions with viruses. Adenovirus and parvovirus lack a lipid bilayer around the virus capsid. Covid-19 disease affects the erythrocyte without entry of SARS-CoV-2 into the erythrocyte. Abbreviations are explained in the text.

Blood group O favors rosetting less than the A or B blood groups. Interestingly, the same pattern can be seen in Covid-19, where blood groups A or B are risk factors, whereas group O appears to be comparatively protective [26]. Glycophorin A is the most abundant sialo-glycoprotein on erythrocytes (**Figure 1**). Sialic acid residues are often used as receptors for invasion by bacteria and other pathogens [27] (**Figure 2**). Sequence analysis of glycophorin A from several primate species revealed high diversifying selection in the glycophorin A gene [27]. High selective pressure could be explained as a consequence of targeting of different pathogens by the glycophorin A receptor. This led to the pathogen decoy hypothesis, which states that the erythrocyte functions as a "flypaper", using glycophorin A to bind pathogens [27]. Pathogens may then be cleared by macrophages as erythrocytes pass through the spleen.

3. The erythrocyte and virus

The erythrocyte does not contain a nucleus or other organelles necessary for virus replication and survival. Its interaction with viruses is therefore mainly restricted to adherence of virus to the plasma membrane, transmembrane proteins or glycocalyx carbohydrates like sialic acid (**Figure 2**). Erythroid progenitor cells may however be infected with parvovirus B19 [28] which may lead to anemia. The ongoing Covid-19 pandemic has renewed interest in erythrocyte-virus interactions, due to the hypoxia or acute respiratory distress syndrome (ARDS) often seen in the disease [29]. Erythrocytes from Covid-19 patients showed increased protein degradation and glycolysis [29]. Increased or decreased levels of many lipids were seen [29]. Some signs of oxidative stress was noted, such as increased oxidized glutathione and increased protein levels of peroxiredoxin 2. Interestingly, N-terminal oxidation or degradation of band3 was noted (**Figure 2**). The cytosolic, N-terminal part of band3 has been shown to be of importance in signaling and hemoglobin regulation [30] and its oxidation or degradation may therefore explain some of

the other noted effects of SARS-CoV-2 on the erythrocyte (**Figure 2**). Based on metabolic and molecular pathway analysis [29], protein degradation pathways, such as proteasome and ubiquitinylation components were identified as significantly affected in erythrocytes of Covid-19 patients (**Figure 2**). In addition, effects were noted on ferroptosis, cyclic-adenosine monophosphate (cAMP) and AMP-activated protein kinase signaling cascades. Lipid metabolism was also affected, especially acyl-carnitine and sphingolipid metabolism [29]. The effect on ferroptosis was based mainly on increased levels of peroxidated lipids and increased levels of heat shock protein beta-1, which inhibits ferroptosis by reducing intracellular levels of iron and maintaining levels of reduced glutathione [31]. The precise mechanism may be through inhibition of transferrin-receptor mediated iron uptake via stabilization of the cytoskeleton [31]. Ferroptosis is an iron-dependent non-apoptotic version of regulated cell death. It is caused by increased levels of iron accompanied by reduced activity of the selenium-dependent enzyme glutathione-peroxidase 4 and mitochondrial changes such as reduced mitochondrial size and mitochondrial membrane rupture. Protein levels of glutathione peroxidase 4 were unchanged in erythrocytes of Covid-19 patients [29]. The absence of mitochondria in the erythrocyte would argue against the existence of ferroptosis in the erythrocyte. In the Covid-19 infected patients, there were no alterations of directly clinically relevant hematological parameters, such as erythrocyte count, hematocrit, or mean corpuscular hemoglobin concentration [29]. The reason for hypoxia or ARDS in Covid-19 patients may therefore not be found in the erythrocyte, possibly with exception of the severe hypoxia without impaired lung function that has been seen in some patients [32]. Respiratory failure is the most common direct cause of death in Covid-19 patients [33, 34]. Studies have reported the nuclear remnants called Howell-Jolly bodies, or seemingly fully nucleated erythrocytes in Covid-19 patients [35, 36]. Nucleated erythrocytes indicate a bad prognosis and often occurs in critically ill patients in several diseases [37]. Mortality risk in Covid-19 seems to be associated with higher erythrocyte distribution width (RDW), a measure of heterogeneity of erythrocyte volume (**Figure 2**). In a meta-analysis of Covid-19 patients, higher RDW was associated with more severe disease [38]. The precise reason for this is unknown, but may involve reduced erythropoiesis or impaired erythrocyte clearance. A more prominent role of erythrocytes in the disease progression can therefore not be ruled out. For instance, in post-mortem analysis of Covid-19 patients with kidney injury, intact erythrocytes were found obstructing peritubular and glomerular capillary lumens [39]. A study of blood samples from Covid-19 patients showed depletion of several immune factors, among them interferon-alpha [40], an interferon that was previously localized in erythrocytes [9]. However, erythrocyte cytokine levels have so far not been analyzed in Covid-19 patients. Anemia in Covid-19 patients may be related to the increased interleukin-6 levels (**Figure 2**) that have been reported in the disease [41]. Interleukin-6 downregulates the iron exporter ferroportin via hepcidin [42]. Lower ferroportin activity makes iron stored in macrophages less available for erythropoiesis and for existing erythrocytes [43, 44]. Increased levels of interleukin-6 have also been implicated as being responsible for the cytokine release syndrome seen in some Covid-19 patients, particularly those affected by ARDS or severe hypoxia [41, 45]. The interleukin-6 receptor blocker tocilizumab showed disappointing results in Covid-19 patients [46, 47] suggesting a more complex pathophysiology of cytokine release syndrome in Covid-19 patients. In HIV-1 patients, anemia often appears together with secondary infections or nutritional deficiencies [44, 48], or is caused by treatment with zidovudine, which is a known cause of bone-marrow suppression [48]. Reduced erythropoietin levels in HIV-1-related anemia may have been caused by upregulation of proinflammatory cytokines like interleukin-1-beta and TNF-alpha [49] or by

autoantibodies to erythropoietin [50]. HIV-1 may infect hematopoietic progenitor cells, but this is not considered a critical cause of anemia in HIV-1 patients [51].

Using biophysical modeling, band3 was suggested to be the point of attachment for SARS-CoV-2 on the erythrocyte surface [32]. The oxidation or degradation noted in band3 [29] may therefore have been caused directly by the binding of the virus. It should be noted that no entry of SARS-CoV-2 into the erythrocyte has as yet been proven (**Figure 2**). The effect of the virus on the erythrocyte may hence be due only to binding to surface receptors. The angiotensin-converting enzyme 2 (ACE2) membrane protein that is considered necessary for entry does not occur in erythrocyte membranes according to the erythrocyte proteome database (http://rbcc.hegelab.org/) [52], making ACE2 an unlikely entry or attachment point for SARS-CoV-2 on the erythrocyte surface [32]. Basigin, one of the erythrocyte receptors for *P. falciparum*, is not considered a receptor for SARS-CoV-2 [53], although such claims have been advanced [54]. The differential susceptibility conferred by the ABO blood group system to Covid-19 may imply a participation of the ABO glycosylation in attachment of the virus to the erythrocyte or other cells [26]. Band3, the suggested erythrocyte attachment protein for SARS-CoV-2, contains ABO glycosylation on the N-642 asparagine residue in the extracellular loop between transmembrane helices TM7 and TM8 [30]. This does not by itself necessarily imply involvement of the erythrocyte in Covid-19 pathogenesis since the ABO blood group system is expressed also on other membrane proteins and in other cells in the human body [55]. In contrast, the ABO blood group system seems not to be of importance for infection by the HIV-1 virus [56].

The erythrocyte has been suggested to be a hiding place for viruses, although these would not be able to infect the erythrocyte. Adsorption to erythrocytes has been shown for HIV-1, influenza and adenovirus [57–59] (**Figure 2**). Zika virus has been shown to survive in erythrocyte samples [60]. Zika virus could be detected in erythrocytes for a significantly longer time than in serum. Considering the ongoing SARS-CoV-2 pandemic, it would be of interest for applied transfusion and blood bank science to know the persistence of the SARS-CoV-2 virus on erythrocytes.

4. Erythrocyte selenium, metal ion and trace element status in relation to virus exposure

Metal ions, trace elements and other compounds contribute in different hierarchies to complex reactions that maintain blood homeostasis (**Figure 3**) [61]. Erythrocyte redox homeostasis is maintained through ascorbic acid, superoxide dismutase, catalase, glutathione and glutathione peroxidase. Antioxidant activity of selenium-dependent glutathione peroxidase may protect band3 of erythrocytes. Selenium is therefore important even for the basic oxygen and carbon dioxide exchange performed by the erythrocyte. Selenium as organic or inorganic form is metabolized by erythrocytes, platelets and neutrophils in different ways [62]. In one study, selenium was supplied together with plain food as inorganic sodium selenite at 200 microgram/day for one year. Neutrophils accumulated selenium more than platelets followed by erythrocytes [62]. In a similar study where selenium was supplied as 1-selenomethionine at 50 microgram/day for one year, platelets accumulated most selenium followed by erythrocytes and neutrophils [63]. These observations indicate that different cells metabolize inorganic and organic forms of selenium differently. The absence of nucleus and organelles in erythrocytes may partly explain the difference. Selenium supplied as inorganic sodium selenite was associated with decreased iron concentration in platelets and neutrophils.

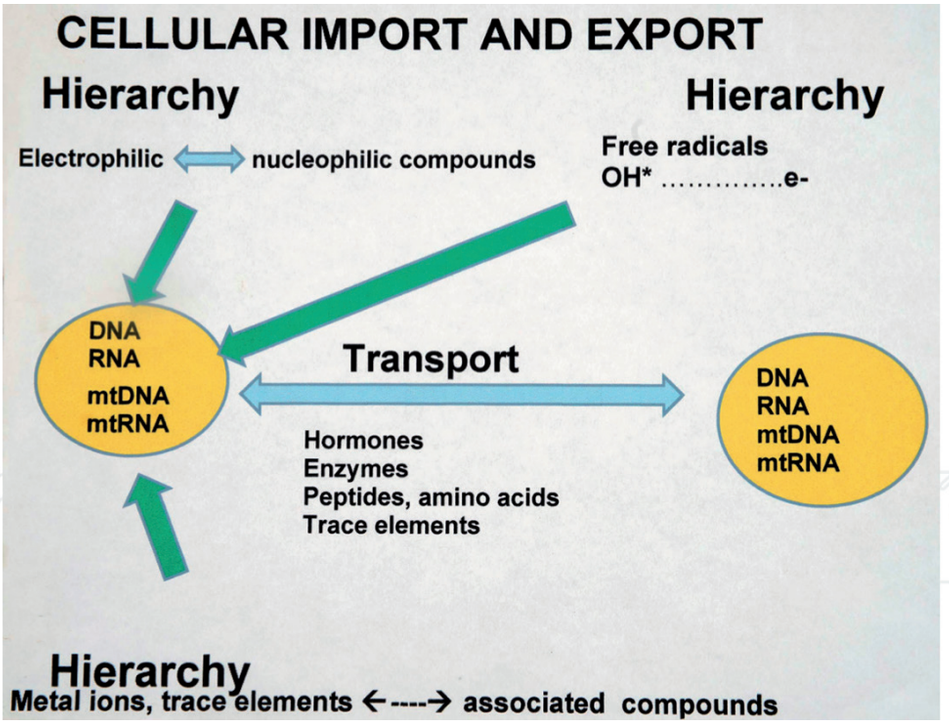

Figure 3.
The flow of compounds and metal ions between cells is dependent on the three main chemical pathways in a hierarchy. The first path involves free radical-induced production of compounds where hydroxyl radical and solvated electron reactions may be involved. The second path involves electrophilic and nucleophilic compounds forming products adequate to the cells. The third path involves metal ions and ligands dependent on the previous two pathways but adapted to cell demand. In erythroid precursors and in other cells, excluding the erythrocyte, compounds and metal ions may reach DNA and RNA not controlled by evolutionary developed genome adapted to cell demand. Small changes of DNA and RNA may take place, an epigenetic change, which may be restored or adapted, but large damages will present symptoms and are difficult to restore.

This result may indicate an iron-regulating function of selenium which may be particularly relevant for the erythrocyte considering its high iron content. Analysis of selenium and other elements was performed by micro-PIXE (micro-particle induced X-ray emission). Increased levels of Cd, Pb and Ag were reported in a study of erythrocytes in patients with Alzheimer's or Parkinson's disease, using the more sensitive and selective ICP-MS (inductively coupled plasma mass spectrometry) [61].

In view of these observations it is interesting that Covid-19 patients with higher selenium and selenoprotein P levels had lower mortality risk [64]. A correlation has also been found between cure rate and hair selenium concentration in Chinese Covid-19 patients [65]. As virus bind to erythrocytes, virus can be transported to different organs e.g. lung, kidney and brain. Viral particles may then find their way through the blood–brain-barrier or enter through the choroid plexus, an important part of the brain filtration system. The SARS-CoV-2 virus may decrease oxygen supply when attached to erythrocytes and impair brain oxygen supply. Low selenium intake may worsen the disease progress.

Erythropoiesis is partly regulated by microRNA and dependent on a high selenium status [66]. For instance, using a mouse loss-of-function allele, microRNA-142 was found to be required for maintaining typical erythrocyte shape, for normal metabolism of reactive oxygen species, and for overall erythrocyte lifespan [67]. MicroRNAs are differentially expressed depending on selenium status [68]. However, a precise role of selenium in regulating microRNA in relation to erythropoiesis has not been described. In addition, mechanisms underlying much of microRNA function are still not fully understood.

5. The erythrocyte and reactive oxygen species

Reactive oxygen species (ROS) are a set of oxygen containing compounds that easily react with and damage other constituents of the cell. Typical ROS in biological systems are superoxide, hydrogen peroxide and hydroxyl radical. ROS can be thought of as forming a hierarchy in the living organism (**Figure 3**). In the erythrocyte, superoxide is produced when Fe(II) of hemoglobin donates an electron to an oxygen molecule, generating the anionic superoxide and the oxidized Fe(III). This reaction is unavoidable due to the existence of iron and oxygen in close contact in the erythrocyte [69]. Superoxide can also be generated in the erythrocyte by nitric oxide synthase, an enzyme identical to the endothelial nitric oxide synthase (e-NOS). The superoxide is converted to water and hydrogen peroxide by copper and zinc-dependent superoxide dismutase. Hydrogen peroxide needs to be further metabolized to avoid the Fenton reaction which otherwise would generate the hydroxyl radical, a very reactive form of ROS. To defuse hydrogen peroxide the erythrocyte contains several enzymes, the most important being catalase, peroxiredoxin 2 and glutathione peroxidase 4. Peroxiredoxin 2 is one of the most abundant proteins in the erythrocyte cytosol [70]. In the oxidized form, peroxiredoxin 2 is recycled by thioredoxin using reduced nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase. Peroxiredoxin 2 is suggested to function as a non-catalytic scavenger of low-level hydrogen peroxide generated from autoxidation of hemoglobin [71]. However, a fraction of peroxiredoxin 2 is found attached to the erythrocyte plasma membrane and may be particularly important in detoxifying membrane lipid peroxides [72]. Catalase and the selenium-dependent glutathione peroxidase are important for the detoxification of hydrogen peroxide received by the erythrocyte from other parts of the vascular compartment. In plasma, superoxide is generated by xanthine oxidase, NADPH oxidase and nitric oxide synthase [73], enzymes that can occur in association to glycosaminoglycans of the endothelial cells of the vascular wall. Superoxide generated by these means can enter the erythrocyte by anion channels like band3. Alternatively, superoxide in plasma may be converted to hydrogen peroxide by extracellular superoxide dismutase. Hydrogen peroxide can then enter the erythrocyte by diffusion through the plasma membrane. Under stress conditions the erythrocyte receives much ROS generated by other cells inside or outside of the vascular compartment. Such ROS may have been generated by mitochondrial stress as a result of bacterial and viral infections (**Figure 3**). For instance, mitochondrial generation of ROS has been proposed as an important part of Covid-19 disease [74]. Antioxidant defense in erythrocytes also depends on small-molecule antioxidants like alpha-tocopherol, glutathione, ascorbate and reductants like NADPH generated in the pentose phosphate pathway. Alpha-tocopherol is present in erythrocyte plasma membrane where it may prevent lipid peroxidation. Glutathione participates as reductant in enzyme-catalyzed reactions. Ascorbate then recycles oxidized forms of alpha-tocopherol and glutathione back to the reduced forms. Superoxide can also react with nitric oxide generating peroxynitrite, a reactive nitrogen species [73]. The extent to which this reaction occurs depends on the presence of nitric oxide inside the erythrocyte. Nitric oxide is produced by nitric oxide synthase but also scavenged by oxyhemoglobin in the erythrocyte. Peroxynitrite can also be formed in plasma and may enter the erythrocyte as anion through band3. In conditions of stress, such as infection with SARS-CoV-2, ROS overload damage the erythrocyte plasma membrane which can lead to hemolysis. The necessity of the antioxidant system of erythrocytes has been proven in gene knock-out studies in mice. For instance, deletion of peroxiredoxin 1 or peroxiredoxin 2 led to increased plasma ROS, hemolytic anemia and shortened life and erythrocyte lifespan [75, 76].

6. The erythrocyte in sepsis

Sepsis is a strong and dysfunctional inflammatory response to an infection. In later stages it proceeds to immunosuppression, decreased pro-inflammatory cytokine levels and increased apoptosis of immune cells. The immunosuppressed stage can last for many months or even years and comes with increased risk for death due to secondary infections. Many aspects of sepsis have been highlighted recently like the presence of exosomes [77], complement and histones [78], metabolic changes [79] and ROS and inflammasomes [80]. Gene expression analysis of sepsis patients revealed CXCL8, tumor protein P53 and TNF as particularly important based on a subsequent pathway analysis [81, 82]. The chemokine CXCL8 binds to chemokine receptors on neutrophils, ultimately leading to attachment of neutrophils at sites of infection. Neutrophils have an important function in phagocytosis of bacteria through neutrophil extracellular traps (NET). Analysis of plasma protein levels of sepsis patients identified the cytokines interleukin-17 and interleukin-27 as being elevated early in sepsis, whereas interleukin-33 protein levels increased later in the immunosuppressive phase [83]. The three interleukins form an axis in sepsis pathophysiology with interleukin-27 having a inhibitory effect on the proinflammatory interleukin-17. The effect of interleukin-27 on interleukin-33 may depend on the stage of disease. Interleukin-33 has been shown to improve survival in murine sepsis models, which can be explained by increased neutrophil presence and more bacterial clearance at the site of infection. Interleukin-33 may have a protective effect by rebalancing different types of immunity [83]. Interleukin-33 has also been reported to suppress and modulate lymphocytes of the innate immune system such as type 2 innate lymphoid cells [84], and induce regulatory T cells. The contribution of erythrocytes to the pool of cytokines in sepsis has not been investigated. However, some of the cytokines implicated in sepsis pathophysiology like interleukin-17, interleukin-33 and CXCL8 have been reported to occur on erythrocytes from healthy individuals [9]. Pathophysiology of sepsis in individuals negative for the CXCL8 receptor Duffy or Duffy knock-out mice has not been reported. Apoptosis is a contributing factor to immunosuppression in sepsis [85]. A first hint of this was the finding that overexpression of B-cell lymphoma 2 (Bcl-2) protected against sepsis [86]. Bcl-2 is a mitochondrial protein that counteracts apoptotic processes. However, in the inflammatory phase of sepsis, some apoptosis of neutrophils is necessary [87].

Changed erythrocyte features have been reported in sepsis, such as the thorny erythrocytes called echinocytes [88] and increased RDW [89]. Reports have also claimed RDW to be of prognostic value in sepsis [90], but this has not been confirmed in other studies [91]. Erythrocytes seem to experience oxidative stress in sepsis [88, 89, 92]. Malondialdehyde, a product of lipid peroxidation, was increased by 3-fold in erythrocytes of sepsis patients [92]. Peroxynitrite has been reported as the major ROS in plasma of sepsis patients [88]. Sepsis patients often suffer from a moderate level of anemia [93]. Anemia in sepsis can have multiple causes such as anemia of chronic disease or hemolysis by bacterial toxins [43]. Hemolysis has often been reported in sepsis caused by *Clostridium perfringens* [94]. It is caused by disruption of the erythrocyte plasma membrane by phospholipase C activity of the *Clostridium* alpha-toxin [94]. A murine cecal ligation and puncture model of sepsis showed that sepsis is lethal in mice deficient for the heme-degrading enzyme heme oxygenase-1 [95]. The same study also showed that administration of the heme-sequestering protein hemopexin could prevent lethality. This suggests the importance of hemolysis in the pathogenesis of sepsis.

Sepsis can also be induced by virus [96]. For instance, COVID-19 and sepsis share similarities but also show some differences. Cytokine storm occurs in both

diseases, although more so in the early stages of sepsis [97]. Lymphopenia has been reported in Covid-19 and is a typical feature of the immunosuppressed later stage of sepsis. Activation of the coagulation cascade occurs in both diseases, including reports of disseminated intravascular coagulation [98]. Similarities in pathophysiology implies that the same therapeutics may be relevant for both diseases. A murine model of sepsis based on cecal ligation and puncture recently showed increased survival with subcutaneous human interleukin-7 [99]. Positive results were later reported from a phase 2 clinical trial [100] of sepsis patients. Based on these results, interleukin-7 therapy has also been suggested for Covid-19 [97]. Interleukin-7 stimulates hematopoietic stem cells to differentiate into the common lymphoid progenitor. The rationale for interleukin-7 therapy is therefore a rebalancing of lymphoid cells, which is implicated by lymphopenia in both diseases. Interleukin-7 may also prevent apoptosis of immune cells [101]. Unfortunately, no approved sepsis-specific therapy exists despite many clinical trials.

7. Protein interactions and signaling mechanisms of possible immunological relevance

Band3 is the most abundant protein of the erythrocyte plasma membrane. The function of band3 is the excretion of bicarbonate formed from CO2 by carbonic anhydrase. A chloride ion is simultaneously imported. The process is reversed in the lungs where CO2 is released. Band3 has been implicated in intracellular signaling based on its cytosolic N-terminal domain, which consists of the first 360 of the 911 amino acid residues of the protein [30]. It was shown to interact with deoxyhemoglobin and enzymes of the glycolytic pathway. The much shorter C-terminal tail (amino acid residues 873–911) interacts with carbonic anhydrase II (CA) (**Figure 4**), forming a metabolon [102]. Band3 also interacts with ankyrin and spectrin [103], which stabilizes the band3 tetramer structure, calnexin during its maturation [30] and stomatin (**Figure 4**), which modulates the anion exchange activity of band3 [104]. Band3 often occurs in complex with glycophorin A presumably contributing to stabilization of the band3 tetramer in the plasma membrane [30]. In stored blood, band3 was found to interact also with flotillin-2, alpha-adducin and adenylosuccinate lyase [15].

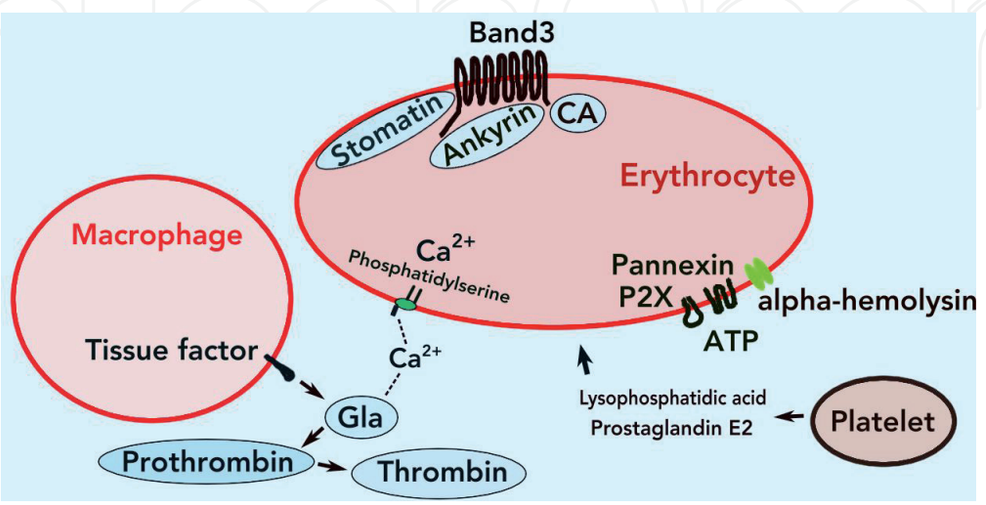

Figure 4.
Schematic figure of protein interactions and signaling mechanisms of the erythrocyte. Abbreviations are explained in the text.

A connection between blood coagulation and the immune system was shown by the finding that macrophages produce tissue factor, a transmembrane protein that starts the extrinsic coagulation cascade [105]. A connection between erythrocytes and coagulation is suggested by a correlation between decreased hematocrit and longer bleeding times. Erythrocytes receive signals in the form of lysophosphatidic acid and prostaglandin E2 from activated platelets [106] (**Figure 4**). Increasing cytosol calcium concentration then increases interaction forces between erythrocytes, thereby contributing to thrombus formation [107]. Increasing intracellular calcium levels also lead to phosphatidylserine exposure on the plasma membrane of the erythrocyte (**Figure 4**). Phosphatidylserine then acts as a binding surface for gamma-carboxyglutamyl residues of the vitamin K-dependent carboxylation/gamma-carboxyglutamic (Gla) domains of coagulation factors [108] (**Figure 4**). This initiates the formation of thrombin from prothrombin [109]. Contribution to coagulation also comes from erythrocyte micro-vesicles that similarly expose phosphatidylserine on the surface. However, erythrocyte micro-vesicles negatively regulate coagulation by supporting activated Protein C [110].

Erythrocytes respond to adenosine nucleotides by the adenosine tri-phosphate (ATP)-gated P2X and P2Y cation channel receptors in the erythrocyte plasma membrane. ATP is released from erythrocytes in an autocrine or paracrine mechanism of action in response to bacterial exotoxins called hemolysins [111]. They are produced by many bacteria and display several mechanisms of action. Alpha-hemolysin of *Escherichia coli* cause an increase in cellular calcium levels and opening of potassium and chloride ion channels [112]. ATP release then occurs either through pannexin channels (**Figure 4**) or through pores formed by hemolysins in the erythrocyte plasma membrane [111]. The subsequent activation of P2X receptors leads to exposure of phosphatidylserine on the surface of erythrocytes, making possible clearance by macrophages. Phosphatidylserine exposure can be prevented by P2X receptor blockers [112]. Purinergic signaling may be a way to avoid the damaging effects of intravascular hemolysis that would result without phosphatidylserine exposure and subsequent clearance by macrophages [112].

8. Senescence, aging, eryptosis and hemolysis of the erythrocyte

The erythrocyte has an average lifespan of around 120 days. As erythrocytes age they become less deformable, shrink in size and display dysfunctional hemoglobin accumulations. Calcium regulation may be part of what determines erythrocyte lifespan (**Figure 5**). Normally calcium concentrations are much lower in erythrocyte cytosol than in the surrounding fluid. During erythrocyte aging, intracellular levels of calcium increase. The events leading to this increase are not fully understood. One proposed mechanism is activation of the mechanosensitive PIEZO1 ion channel [113] through decreased deformability of the aging erythrocyte (**Figure 5**). PIEZO1 is a rather unspecific ion channel and can therefore cause general dissipation of ion gradients. In the case of calcium this would mean higher intracellular concentration. Gradually dysfunctional plasma membrane calcium efflux channels may also contribute to the higher calcium levels. Higher intracellular calcium concentrations are known to activate the Gardos channel, a potassium efflux channel in the plasma membrane involved in the regulation of cell volume in erythrocytes (**Figure 5**) and some other cells. Activation of the Gardos channel leads to potassium export and concomitant loss of water and chloride, together known as the Gardos effect. As they grow older, erythrocytes accordingly become more dehydrated. Support for this also comes from mutations in PIEZO1 [113] and the Gardos channel.

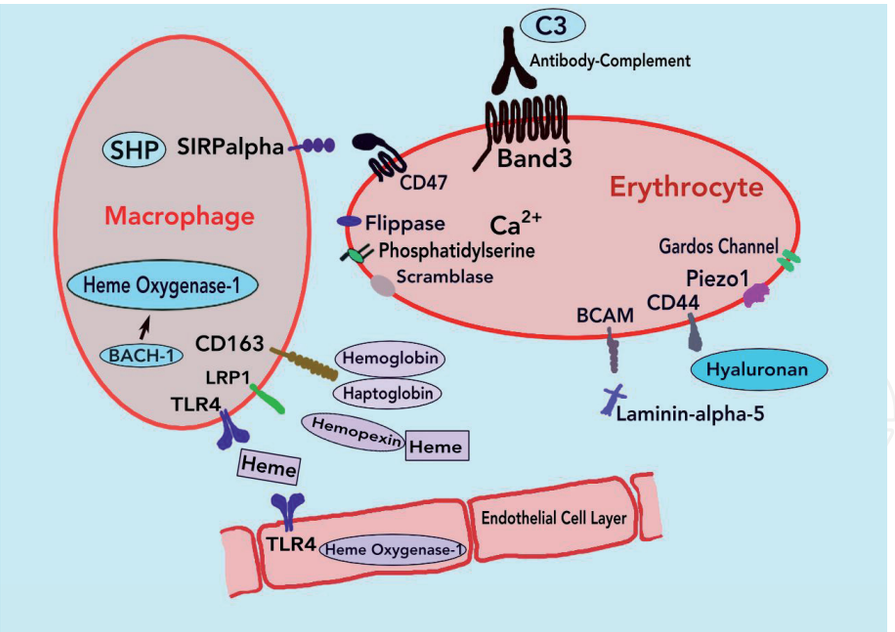

Figure 5.
Schematic figure of senescence, aging, eryptosis and hemolysis of the erythrocyte. Abbreviations are explained in the text.

The sequence of events from dehydration to erythrocyte clearance from circulation have not been fully elucidated. In one recently proposed model, erythrocytes may respond to dehydration by shedding Glycophorin C-containing vesicles, thereby losing membrane sialic acid [13]. Less sialic acid would result in activation of the basal cell adhesion molecule (BCAM, also known as Lutheran antigen) and CD44 membrane proteins of the erythrocyte. Ligands of BCAM and CD44 are laminin-alpha-5 and hyaluronan (**Figure 5**), known as parts of the extracellular matrix. Binding to laminin-alpha-5 or hyaluronan could possibly delay erythrocyte passage through spleen and liver making encounters with macrophages and clearance from circulation more likely.

Activation of the Gardos channel and dehydration are thus effects of increased erythrocyte calcium levels. Another consequence of increased calcium levels is phosphatidylserine exposure on the erythrocyte plasma membrane (**Figure 5**). This effect is likely to occur because of calcium-caused activation of scramblase, a membrane enzyme that equilibrates membrane lipids between the two plasma membrane leaflets. Further to this effect, calcium inactivates flippase, a membrane enzyme that moves lipids from the outer plasma membrane to the inner plasma membrane leaflet. Exposed phosphatidylserine can then bind to several phosphatidylserine receptors exposed on phagocytic cells [114], facilitating clearance of erythrocytes from circulation by spleen or liver macrophages. In young and healthy cells, phosphatidylserine occurs mainly on the inner leaflet of the plasma membrane. Exposure of phosphatidylserine is a marker for cell stress and effectively functions as an "eat-me" signal.

Yet another contribution to erythrocyte aging comes from the membrane protein CD47, a heavily glycosylated protein with five transmembrane domains and an extracellular N-terminal immunoglobulin-like domain (**Figure 5**). CD47 is a "don't eat me" signal that protects the cell from being phagocytosed by macrophages or other phagocytes. CD47 binds the macrophage signal-regulatory protein alpha (SIRP-alpha), a transmembrane protein with three immunoglobulin-like extracellular domains. CD47-SIRP-alpha interaction leads to phosphorylation of immunoreceptor tyrosine-based inhibition (ITIM) motifs in the cytoplasmic tail of SIRP-alpha. This leads to the recruitment of Src homology phosphatases (SHP) preventing

myosin-IIA accumulation and phagocytosis by the macrophage. As erythrocytes grow older they display less CD47 on their surfaces, thereby increasing the likelihood for being eaten by macrophages. However, the role of CD47 in erythrocyte clearance may not be limited to a reduced "do not eat me" signal. CD47 can also bind thrombospondin-1, a multifunctional protein of the extracellular matrix. Evidence suggests that conformational changes in CD47, perhaps caused by oxidation as the cell ages, promotes binding of thrombospondin. The resulting complex then instead becomes an "eat me" signal when binding to SIRP-alpha of the macrophage [115].

Senescence and aging has been suggested to be regulated through band3 acting as a "molecular clock" for the erythrocyte [116]. Partial proteolysis, oxidation, phosphorylation and binding of methemoglobin or hemichrome to band3 leads to protein clustering and conformational changes that are recognized by anti-band3 autoantibodies (**Figure 5**). Binding of C3 complement then leads to phagocytosis of the aging erythrocyte when it encounters red pulp spleen or liver macrophages (**Figure 5**). Presumably, antibodies and complement must reach a threshold of opsonization for clearance by macrophages to take place. An appealing aspect of this "clock" is that it gauges the content of dysfunctional hemoglobin inside each erythrocyte, thus weeding out erythrocytes that have become unacceptably dysfunctional in their crucial role as oxygen carriers. If erythrocytes are ruled by a "clock", another candidate is the period circadian protein homolog 2 (PER2) of the circadian clock, a physiological system that determines the biological day-length of the organism. Erythrocytes of PER2-deficient mice showed morphological changes and impaired oxygen transport [117].

Eryptosis is the regulated cell death associated with erythrocytes. Eryptosis can be induced by oxidative stress, pathogen infection, certain diseases like sepsis and sickle-cell disease and certain drugs and chemicals. Eryptosis can also be protected against by several means, notably some plant-derived substances like ascorbate, caffeine and resveratrol [118]. Signaling leading to eryptosis involves ceramide, prostaglandin E2 and increased intracellular calcium levels. Similar to what happens in senescence and aging, this induces scramblase and inhibits flippase leading to phosphatidylserine exposure on the plasma membrane. Eryptosis is largely seen as a means for the organism to avoid the serious consequences of hemolysis and may even prevent pathogen growth [118]. Still, phosphatidylserine receptors on vascular endothelial cells can bind eryptotic erythrocytes leading to impaired microcirculation [119]. Regulation of eryptosis involves several kinases like the p21-activated kinase PAK2 and mitogen-and stress-activated kinase MSK1/2 [119]. Experiments with knock-out mice further suggested involvement of the beta-glucosidase-like protein Klotho. In healthy human and mice, Klotho is a co-receptor for fibroblast growth factor 23, regulating phosphate metabolism and vitamin-D synthesis. Klotho deletion ultimately leads to premature aging and death. Whether Klotho is involved in regulation of physiological eryptosis in humans remains unknown.

Both eryptosis and senescence involve erythrocyte-macrophage interactions. The close interactions between erythrocytes and macrophages follow throughout the erythrocyte lifespan. To begin with, macrophages participate in erythropoiesis, the formation and development of erythrocytes in the erythroblastic islands of the bone marrow. During the lifetime of the erythrocyte, passage through spleen and liver generates new macrophage encounters. In these encounters erythrocytes are screened for healthiness and may be repaired by the macrophages [120], for instance by removal of the inclusion bodies containing damaged hemoglobin, called Heinz bodies. Erythrocytes are the main source of blood MIF1 (macrophage migration inhibitory factor 1), although the physiological significance of this remains to be established [8]. At last, the senescent or eryptotic erythrocyte is phagocytosed by spleen or liver macrophages.

Hemolysis is the lysis of erythrocytes and release of their cellular contents in surrounding fluids. Intravascular hemolysis occurs mainly as a consequence of bacterial infection and production of endotoxins. Hemolysis may also occur as a consequence of shear stress when erythrocytes pass through narrow capillaries. The mechanism underlying hemolysis is overhydration due to defective ion efflux channels, particularly sodium/potassium ATPase. Cell volume increase will lead to rupture of the cell membrane and release of cytosolic contents. Hemolysis is typical of some diseases such as sickle-cell disease or glucose-6-phosphate deficiency. The fate of hemoglobin is an important aspect of hemolysis. Free hemoglobin typically binds to haptoglobin and the complex is then internalized through binding to the CD163 receptor on macrophages (**Figure 5**). If this does not occur, and if the iron has been oxidized to the ferric Fe(III) state, plasma hemoglobin will be separated into globin and heme. Being hydrophobic, free heme makes contact to plasma proteins such as albumin, alpha-1 antitrypsin, alpha-2 macroglobulin, lipoproteins and hemopexin. Binding to hemopexin effectively sequesters heme, making the complex largely safe for further circulation in the vascular compartment. The complex will eventually be internalized through binding to the low density lipoprotein receptor-related protein 1 (LRP1) receptor (**Figure 5**) on hepatocytes or macrophages [121]. If bound to albumin, heme can be transported into cells by binding to transferrin receptor 1. If cellular uptake does not happen by these routes, for instance due to hemopexin saturation, free heme can contribute to inflammation by binding the TLR4 receptor (**Figure 5**). Heme is in this context acting as a danger-associated molecular pattern (DAMP). Since TLR4 occurs also on endothelial cells, the vasculature is able to directly respond to increased heme levels. The effect of TLR4 stimulation is activation of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), inflammasome activation leading to activation of caspase-1 and release of pro-inflammatory interleukins like interleukin-1-beta, and interleukin-18. Recently, binding of heme to TLR4 was shown to be dependent on myeloid differentiation factor 2 (MD-2) and the glycosylphosphatidyl inositol (GPI)-anchored leucine-rich repeat protein CD14 [122]. As a comparison, in eryptosis and normal senescence and aging, macrophages typically phagocytose the whole erythrocyte and free heme does not appear.

Heme can also activate the alternative pathway of complement, and activate platelets through C-type-lectin-like receptor 2 [123]. In sickle cell disease patients and murine models of sickle-cell disease, hemolysis caused kidney deposits consisting of complement C3. The condition was probably caused by heme activation of complement, since it was possible to counteract by addition of hemopexin [124]. Free heme and its iron is available for use by bacterial pathogens and this is considered as an important aspect of hemolysis, for instance in hemolytic anemia. Sequestering of heme and hemoglobin is thus an important protection against damage caused by hemolysis. Inside the cell, heme is catabolized by heme oxygenase-1, producing biliverdin, free iron and carbon monoxide. The expression of heme oxygenase-1 can be induced by heme relieving gene repression through the BACH-1 heme-sensing repressor (**Figure 5**). The net effect of the catabolism is largely anti-inflammatory due to lower levels of TNF-alpha, interleukin-1-beta, and macrophage inflammatory protein (MIP) and upregulation of interleukin-10 [125]. The carbon monoxide produced as a by-product may however contribute to oxidative stress due to reactions with reduced transition metals [125]. Heme oxygenase-1 is itself a heme-containing enzyme of the heat shock protein family, present in the endoplasmic reticulum membrane oriented to the cytosol and expressed throughout the body (**Figure 5**). The globin part of hemoglobin is regarded as largely non-inflammatory [3]. Heme and its consequences are the most well-studied aspects of hemolysis. Less well-studied effects of hemolysis include release of intracellular or surface-bound cytokines from erythrocytes.

9. Some genetic diseases particularly relevant in the erythrocyte context

Glucose-6-phosphate dehydrogenase is an enzyme in the glycolysis and pentose phosphate pathways. Its deficiency particularly effects the erythrocyte since the erythrocyte depends on glycolysis and the pentose phosphate pathway for its energy needs. Erythrocyte irregularities called Heinz bodies are frequent in glucose-6-phosphate deficiency. The deficiency is particularly common in sub-Saharan Africa due to a survival advantage in malaria-afflicted areas. Interestingly, deficiency for glucose-6-phosphate dehydrogenase seems to be associated with hemolysis in Covid-19 patients [126]. Patients with glucose-6-phosphate deficiency may react with hemolysis as a response also to other infections and treatment with primaquine or hydroxychloroquine [127, 128], suggesting a more general susceptibility to hemolysis associated with glucose-6-phosphate deficiency. Some other diseases involving the erythrocyte are sickle-cell disease, thalassemia, stomatocytosis, spherocytosis, ovalocytosis, paroxysmal nocturnal hemoglobinuria (PNH), polycythemia vera, acute erythroid leukemia and lecithin-cholesterol acyltransferase deficiency (LCAT). Paroxysmal nocturnal hemoglobinuria (PNH) is an intravascular hemolytic anemia, most often caused by somatic mutations in the gene encoding phosphatidylinositol glycan A (PIGA), leading to dysfunctional GPI-dependent anchoring of membrane proteins. One such erythrocyte membrane protein is decay accelerating factor (DAF) which acts to limit action of the alternative complement pathway. The resulting complement-driven hemolysis can be counteracted by the humanized monoclonal antibody eculizumab directed against terminal complement component C5.

10. Outlook for new treatments and diagnosis

The Duffy antigen may be of diagnostic value for some diseases, particularly malaria, but may perhaps also prove to be a druggable target, since G-protein coupled receptors are among the most common drug targets. The signaling mechanisms discovered recently particularly regarding band3 may also be of pharmacological interest. The mechanism underlying eryptosis may be used to kill off infected erythrocytes [129]. It is known that antioxidants like ascorbic acid and catechin, provided through the diet can have protective effects on the erythrocyte. Refined erythrocyte related diet recommendations could perhaps be expected. The erythrocyte has previously been used in various physical or biochemical ways as a diagnostic tool. Such tests are the erythrocyte sedimentation rate, hematocrit, erythrocyte distribution width, erythrocyte count, mean corpuscular hemoglobin concentration and mean corpuscular volume. New diagnostic or treatment developments can be expected based on molecular, biotechnological and genetic techniques. One example would be ICP-MS to detect element profile changes of genetic, epigenetic or environmental origin.

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