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REVIEW

Red cell DAMPs and inflammation

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Abstract Intravascular hemolysis, or the destruction of red blood cells in the circulation, can occur in numerous diseases, including the acquired hemolytic anemias, sickle cell disease and β-thalassemia, as well as during some transfusion reactions, preeclampsia and infections, such as those caused by malaria or *Clostridium perfringens*. Hemolysis results in the release of large quantities of red cell damage-associated molecular patterns (DAMPs) into the circulation, which, if not neutralized by innate protective mechanisms, have the potential to activate multiple inflammatory pathways. One of the major red cell DAMPs, heme, is able to activate converging inflammatory pathways, such as toll-like receptor signaling, neutrophil extracellular trap formation and inflammasome formation, suggesting that this DAMP both activates and amplifies inflammation. Other potent DAMPs that may be released by the erythrocytes upon their rupture include heat shock proteins (Hsp), such as Hsp70, interleukin-33 and Adenosine 5' triphosphate. As such, hemolysis represents a major inflammatory mechanism that potentially contributes to the clinical manifestations that have been associated with the hemolytic diseases, such as pulmonary hypertension and leg ulcers, and likely plays a role in specific complications of sickle cell disease such as endothelial activation, vaso-occlusive processes and tissue injury.

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Red cells and their disorders

The red cells, or erythrocytes, are the most abundant cells in the blood, where their main function is to transport oxygen and carbon dioxide throughout the organism. These cells are anucleate, biconcave discoids (facilitating gas exchange and membrane deformability, allowing their passage through small vessels) that are filled with hemoglobin, the principal oxygen-binding protein. Red blood cells are produced in the bone marrow via erythropoiesis and released into the circulation, where they usually survive for between 100 and 120 days, although final maturation of circulating reticulocytes (immature nucleated red cells) can also occur in the spleen [1]. In general, erythrocyte destruction and hemoglobin processing occur principally via phagocyting macrophages in the spleen, and also in the blood marrow and liver, without the release of damaging hemoglobin into the plasma (extravascular hemolysis). Of the numerous red cell disorders, the anemias are distinguished by a reduction in red cell volume. In addition to impairments in red cell production, the anemias may also result from augmented erythrocyte destruction, as occurs in the hereditary hemolytic anemias and in acquired hemolytic anemias [2].

Of particular relevance to this review are those disorders that cause excessive red cell destruction in the circulation, or intravascular hemolysis, as the red cells contain a number of damage-associated molecular patterns (DAMPs) that, if released into the plasma, can potentially cause inflammatory mayhem if not neutralized rapidly by the

organism's innate protective mechanisms. Diseases and events that are characterized by elevated intravascular hemolysis include the acquired hemolytic anemias, such as autoimmune hemolytic anemia, paraoxysmal nocturnal hemoglobinuria, mismatched transfusion reactions, preeclampsia and infections, such as those caused by malaria or *Clostridium perfringens* [3, 4] (see Table 1 for a summary of some of the major causes of intravascular hemolysis). Hemolytic anemia can also occur following high-dose intravenous immunoglobulin (IVIG) administration, where non-O blood group patients with underlying inflammatory or immune-mediated disorders are reportedly at increased risk of hemolysis [5].

Some of the hereditary hemolytic anemias (caused by alterations in hemoglobin or alterations in the red cell membrane or its enzymes) can also present augmented intravascular hemolysis, in addition to extravascular hemolysis. Hemolytic anemia is characterized by decreased hemoglobin levels (typically below 10 g/dL, but reaching as low as less than 6 g/dL in cases of severe hemolysis), reduced red blood cell counts and haptoglobin levels, in addition to increased bilirubin levels [6]. Additionally, elevated plasma lactate dehydrogenase (LDH), particularly of the LDH-1 and LDH-2 isoenzymes, has been suggested to be a marker of intravascular hemolysis [6, 7]. Chronic hemolysis often presents as anemia when erythropoiesis cannot match the pace of red cell destruction, leading to reticulocytosis, in addition to manifestations such as jaundice and gallstones that occur as a consequence of hemoglobin and heme catabolism [8]. The hemoglobin disorders, such as sickle cell disease and β-thalassemia, can induce extensive red cell destruction and, therefore, intravascular hemolysis and, although extensively studied, the chronic inflammation that can ensue from these hemoglobin disorders, and indeed hemolytic processes in general, is only just being recognized [9].

Table 1 Major clinical causes of hemolysis

Class of disorder	Examples	Acute/chronic hemolysis
Hemoglobin abnormalities (hemoglobinopathies)	Sickle cell disease	Chronic
	Thalassemias	Chronic
Red cell membrane disorders	Hereditary spherocytosis	Chronic
	Hereditary elliptocytosis	Chronic
Red cell enzyme disorders	Glucose-6-phosphate dehydrogenase (G6PD) deficiency	Chronic/acute
	Pyruvate kinase (PK) deficiency	Chronic
Acquired hemolytic anemias	Paroxysmal nocturnal hemoglobinuria (PNH)	Chronic
	Liver cirrhosis	Acute
	Infections (e.g. malaria, *Clostridial sepsis*)	Chronic/acute
	Preeclampsia/hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome	Acute
	High-dose IVIG administration	Acute
Mechanical/physical trauma	Microangiopathic hemolytic anemia	Chronic
	Abnormalities in heart and large vessels	Chronic
	March hemoglobinuria (due to prolonged marching/foot striking, etc.)	Acute
	Burns	Acute
Chemical lesions	Spider/snake bites/bee stings	Acute
	Drug-induced (usually oxidant drugs)	Acute
	Copper/lead toxicity	Acute
	Osmotic (water/hypotonic injection)	Acute
Alloimmune disorders	Mismatched transfusions	Acute
	Hemolytic disease of the fetus/newborn	Acute
Autoimmune hemolytic anemia (AIHA)	Warm-active autoantibody AIHA (can be associated with some autoimmune disorders, lymphoproliferative disorders amongst others)	Chronic
	Cold-active autoantibody AIHA (may be associated with infections)	Acute
	Drug-induced	Acute

All of these disorders are associated with some degree of intravascular hemolysis; however, the red cell membrane disorders usually present a higher proportion of extravascular hemolysis, rather than intravascular hemolysis. Table compiled using information from [4, 5, 158, 159]

IVIG intravenous immunoglobulin

Sickle cell disease and β-thalassemia

The term sickle cell disease describes a group of genetic disorders caused by mutations in the hemoglobin β chain, resulting in the production of abnormal hemoglobin S (HbS) in the erythrocyte. The most common form of the disease is homozygotic HbSS disease (sickle cell anemia; SCA), although other genotypes in which one sickle gene is co-inherited with another mutation that alters the β-globin chain also exist (for example, HbSC disease and HbS/β thalassemia) and, together with SCA, these are collectively denominated sickle cell disease (SCD) [10].

At low concentrations of oxygen and high hemoglobin concentrations, HbS polymerizes leading the erythrocyte to take on a characteristic sickle-shaped morphology. The pathophysiological mechanisms of SCD are complex, but are now known to also involve intravascular hemolytic processes and recurrent vaso-occlusion, together with chronic vascular inflammation and endothelial activation [11, 12]. These events result in the numerous and varied clinical complications that are associated with SCD, including painful vaso-occlusive episodes, autoinfarction of the spleen, pulmonary hypertension, acute chest syndrome, stroke, end-organ damage, renal damage and a shortened lifespan [13].

In another class of hemoglobinopathies, the β-thalassemias, a reduced or abolished synthesis of the beta-globin chains occurs, causing the precipitation of free α-globin chains and resulting in ineffective erythropoiesis, red cell destruction and, therefore, anemia. Alterations in the red cell membrane cause membrane damage and instability, which can induce phosphatidyl serine exposure and induce hypercoagulability. Splenomegaly is the hallmark of β-thalassemia, although thrombocytopenia and leukopenia can also occur, in addition to an increased requirement for blood transfusions. Splenectomy can be used in the management of some hematological diseases, such as β-thalassemia, to reduce spleen-driven extravascular hemolysis [14]; however, a number of risks and complications are reported to be associated with splenectomy in β-thalassemia, including an increased risk of infection and venous thromboembolism [14], besides a significant shift towards intravascular hemolysis [15, 16].

Vascular inflammation and hemolytic diseases

Increasing evidence emphasizes the participation that inflammatory processes and ensuing vascular dysfunction have in the pathophysiology of many of the hemolytic diseases. In SCD, for example, the chronic inflammatory state associated with the disease drives a vicious cycle of pan-cellular activation, inflammatory mediator release and leukocyte and red cell recruitment to the endothelium, culminating in the occlusion of blood vessels, particularly in the microcirculation. The driving role of inflammation in SCD vaso-occlusive processes has been reviewed in more detail in [17–19] and these mechanisms are summarized in Fig. 1. SCD vaso-occlusive processes are responsible for the acute painful episodes that are a characteristic of the disease and, indirectly, for many of the disease's complications [20]. In addition to other triggering mechanisms, such as repeated events of hypoxia and reperfusion, a clear role for intravascular hemolysis in the initiation of vascular inflammation in SCD is emerging [18].

Chronic blood transfusion of some individuals with β-thalassemia often leads to iron overload and, therefore, low-grade inflammation, endothelial activation and a higher cardiovascular risk [21]. Furthermore, splenectomy (sometimes employed in the management of β-thalassemia) has been reported to significantly increase intravascular hemolysis in hemoglobin E/β-thalassemia individuals, in association with elevations in biomarkers indicative of inflammation, endothelial activation and hypercoagulability [15]. Leg ulcers are relatively frequent in individuals with β-thalassemia, SCD, hereditary spherocytosis and PNH, while incidences of pulmonary hypertension (PH) and priapism have also been reported in all these diseases, suggesting a common role for intravascular hemolysis and consequent vascular dysfunction in these complications [16, 22, 23].

Malaria infections can cause intravascular hemolytic processes [2] that may contribute to the reduced nitric oxide (NO) bioavailability and inflammatory processes associated with complications of the disease. During cerebral and severe malaria due to *Plasmodium falciparum* (the latter being characterized by the dysfunction of the brain tissue and others organ, such as the lungs or kidneys), augmented pro-inflammatory cytokine production, cellular adhesion molecule expression, endothelial activation and decreased NO bioavailability cause the adhesion of red blood cells and leukocytes to the brain and lung vasculature, respectively. In the brain, the congestion of cerebral capillaries leads to blood brain barrier dysfunction and edema, while the margination and subsequent migration of red blood cells, leukocytes and platelets from the lung microvasculature into interstitial tissue and the alveolar air space can lead to the acute lung injury and acute respiratory distress syndrome that can be associated with severe malaria [24].

Red cell DAMPs

The hemolytic process leads to the release of a number of red cell components that are recognized as DAMPs which can trigger a sterile inflammatory response. Erythrocyte

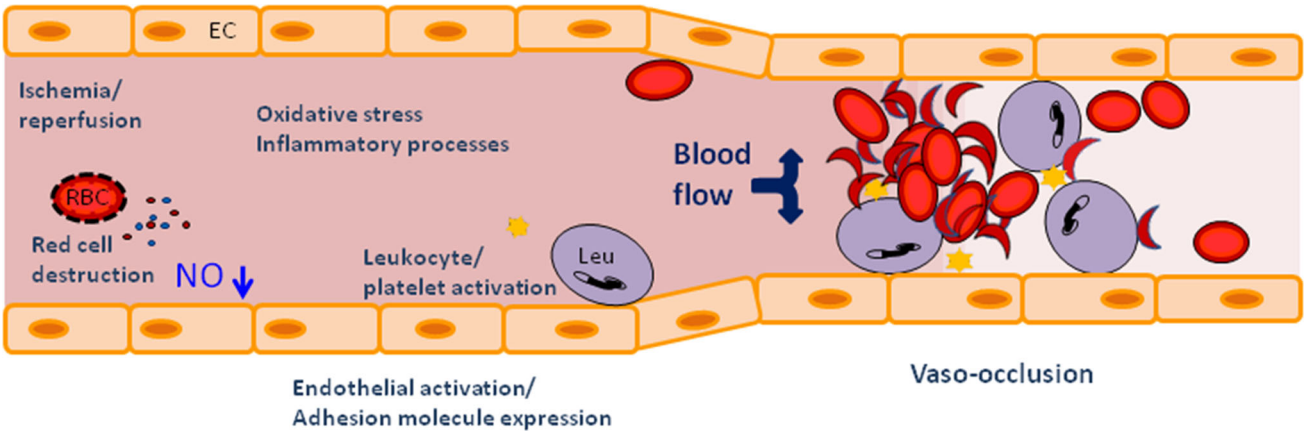

Fig. 1 Inflammation as a driver of vaso-occlusion in sickle cell disease. The production of abnormal hemoglobin S (HbS) leads to a number of pathophysiological events, including intravascular hemolysis and processes of ischemia/reperfusion. Cell-free hemoglobin, released from damaged red blood cells (RBC), reduces nitric oxide (NO) bioavailability (facilitating vasoconstriction) and induces reactive oxygen species (ROS) production. Oxidative stress is also caused by processes of ischemia–reperfusion (I/R) in blood vessels. Inflammatory processes resulting from damage-associated molecular patterns (DAMPs) release from RBC, oxidative stress, abnormalities in the sickle RBC membrane and I/R injury, cause cytokine and inflammatory mediator production, consequently contributing to the activation of endothelial cells (EC), the coagulation system and leukocytes (Leu). The increased expression of adhesion molecules on the surface of these cells leads to their recruitment to the blood vessel wall and a decrease in blood flow. The subsequent increase in RBC transit time can induce RBC sickling and lead to the sequestration of RBC in the cellular agglomeration, consequently inhibiting flow and causing vaso-occlusion. Figure adapted from [160], with permission *yellow star* platelet; *red/blue small circles* red cell DAMPs

components that potentially act as DAMPs following red cell destruction are listed below and in Table 2.

Hemoglobin/Heme

Upon the destruction of the red cells in the blood vessels, a significant quantity of hemoglobin and other contents of the cell are released into the circulation. If this cell-free hemoglobin is not swiftly neutralized by specialized scavenger proteins, it can cause significant damage in the vascular, perivascular and endothelial spaces [25]. In the event of extensive hemolysis, innate protective mechanisms are overwhelmed and decompartmentalized hemoglobin can react with significant quantities of vascular NO via multiple pathways [26]. NO dioxygenation of oxyhemoglobin can generate ferric hemoglobin ($Hb–Fe^{3+}$) and nitrate (NO^{3-}), while iron–nitrosylhemoglobin may also be formed by the direct nitrosylation of the iron of deoxyhemoglobin ($Hb–Fe^{2+}$) [26, 27]. While the S-nitrosation of hemoglobin at the β-cysteine 93 (β-cys93) residue, forming S-nitrosohemoglobin (SNO-Hb) (which can release NO during deoxygenation), and the reduction of nitrite to NO by deoxygenated hemoglobin represent mechanisms for the retention of some of the biological activity of NO in the circulation [28, 29], the immediate bioavailability of vascular NO is essentially considerably decreased during hemolytic processes, potentially augmenting vasoconstriction and altering blood flow. Since NO is also important for maintaining cellular homeostasis, a reduction in its vascular bioavailability can bring about endothelial

Table 2 Known and putative red cell DAMPs and their receptors

Red cell DAMP	Role Function in RBC/source	Associated receptor(s)
Heme	Released following oxidation of cell-free hemoglobin	TLR4 and NLRP3
Hsp70	Molecular chaperone	TLR2, TLR4, CD14
ATP	Energy source, released from RBC during cell damage, hypoxia, reduced oxygen tension and oxidative stress	NLRP3 and P2X7
mt DNA	Residual (from reticulocytes)	TLR9, NLRP3
IL-33	Stored by RBC (?)	ST2
Cyclophilin A	Chaperone found in cytosolic fraction (residual from reticulocytes)	CD147

RBC red blood cell, *TLR* toll-like receptor

dysfunction and platelet activation amongst other effects [30, 31]. More recent data have highlighted the significant effects that intravascular hemolytic processes may have on inflammatory processes. The induction of acute hemolytic events in C57BL/6 mice (using intravascular water infusion, resulting in plasma hemoglobin levels that were similar to those seen in a mice model of SCD) was found to induce an almost immediate and extensive vascular inflammatory response, characterized by extensive leukocyte recruitment to the blood vessel walls of the microcirculation [32].

In addition to its oxidation by NO, hemoglobin can react with physiological oxidants such as hydrogen peroxide and lipid peroxide. The oxidized hemoglobin product, Hb–Fe^{3+}, accumulates in the circulation and tissues [27], where it can release hemin (or heme, as it is commonly termed), a hydrophobic inflammatory molecule [33, 34]. Heme exerts multiple inflammatory effects, activating leukocytes and their migration, upregulating adhesion molecule and cytokine expression and augmenting oxidant production and lipid peroxidation [33, 35–37]. Importantly, heme, but not porphyrins without iron, can act as a DAMP promoting the formation of the inflammasome in LPS-primed macrophages [34], in addition to inducing toll-like receptor (TLR)-4-mediated endothelial cell activation and macrophage TNF-α (TNF)-production [33, 38]. In addition to receptor-mediated effects, heme can bind to transcription factors, such as Bach-1, a transcriptional regulator of heme-oxygenase-1 (HO-1). Due to its hydrophobicity, heme can insert itself in, and impair, lipid bilayers and organelles [39], and also binds to and oxidizes proteins or lipids, to generate reactive molecules, including oxidized low-density lipoprotein, an extremely inflammatory molecule with cytotoxic activity [37]. As a result of such oxidant reactions, heme can induce significant reactive oxygen species (ROS) generation and, therefore, oxidative stress [39, 40]; additionally, receptor-mediated ROS generation may occur via signaling pathways involving protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K) in neutrophils [36]. Heme-induced oxidative stress can, in turn, also upregulate the expression of redox-sensitive genes, such as the cytoprotective HO-1 enzyme [41]. The effects of LPS-mediated activation of NFκB and cytokine production in macrophages can also be potentiated by heme via spleen tyrosine kinase (Syk)-mediated ROS generation, suggesting a role for heme in amplifying the innate immune response to microbial molecules [42]. In macro- and micro-vascular endothelial cells, heme has been shown to induce tissue factor expression in an NFκB-dependent fashion [43], while, more recently, heme-laden erythrocyte microparticle generation has been reported in sickle cell disease (SCD), where these microparticles can adhere to and transfer heme to endothelial cells, inducing oxidative stress and apoptosis [44].

ATP and adenosine

Adenosine 5′ triphosphate (ATP) has vasoactive properties and acts a universal energy source in cellular reactions. ATP release can occur during necrosis, although numerous molecular pathways can result in augmented extracellular ATP. In the case of red cells, ATP can be released following cell lysis and there are reports to indicate that red cells release ATP when subjected to hypoxia and reduced oxygen tension, as well as oxidative stress, as may occur in vaso-occlusive processes, such as those seen in SCD [10, 45–47]. The intracellular ATP concentration in red blood cells is high (millimolar concentrations) [48]; as such, hemolytic mechanisms conceivably contribute to significant elevations in extracellular ATP in the red cell disorders [49].

Extracellular ATP can act as a potent signaling molecule via the activation of purinergic P2 receptors [50], with inflammatory consequences. For example, binding of ATP to the P2X7 receptor on inflammatory cells may contribute to Nod-like receptor family, pyrin domain-containing (NLRP)3 activation and inflammasome formation, due to potassium efflux via ATP-gated cation channel opening [51]. Increased extracellular ATP levels may amplify inflammation in vivo by stimulating leukocyte activation and recruitment via P2 receptor activation [52–54]. Extracellular ATP can also potentially exert important effects on the erythrocytes themselves, as erythrocyte P2 receptor activation on progenitor red blood cells can cause microparticle release, ROS formation and apoptosis, while stimulation of P2 receptors on mature red blood cells can trigger eicosanoid release, phosphatidylserine exposure and further hemolysis [55].

Furthermore, extracellular ATP is converted by ectonucleotidases to adenosine, another vasoactive mediator. Adenosine concentrations have been found to be elevated in the plasma of transgenic SCD mice and humans with SCD [56], probably due to hemolysis, in addition to hypoxia and tissue damage. While the interaction of adenosine with the Adora2a adenosine receptor may have anti-inflammatory effects by selectively inhibiting the invariant natural killer T (iNKT) cells, adenosine signaling through the Adora2b adenosine receptor on the red blood cell membrane can contribute to erythrocyte sickling in SCD [56, 57].

Other red cell DAMPs

Other potential DAMPs that may be released by damaged red cells include some of the heat shock proteins (Hsp).

Hsp70 has been identified in mature erythrocytes [58] and, while Hsp70 has a protective chaperone function, this ATP-driven protein can stimulate monocytes/macrophages, microglia and dendritic cells via the TLR2 and 4 and CD14 pathways, leading to the activation of intracellular signaling pathways [59]. Furthermore, an Hsp70-TLR4 axis has been implicated in albumin-stimulated renal tubular inflammation in mice [60]. In pregnant women with hemolysis, elevated liver enzymes and low platelet count syndrome (HELLP syndrome), elevated serum Hsp70 levels are thought to indicate tissue damage, due to both hemolysis and hepatocellular injury, and disease severity [61]. Upregulated levels of Hsp70 have been reported in the erythrocyte proteome of HbEβ-thalassemia patients [62] and increased levels of circulating serum Hsp70 level have been described in SCD individuals during vaso-occlusive crisis [63].

IL-33 is a nuclear-associated IL-1 family cytokine that signals via the ST2 receptor, inducing innate immune cells to produce type 2 cytokines [64]. A major source of IL-33 may be the red cells, which have been shown to release significant amounts of this cytokine upon their hemolysis. Circulating levels of IL-33 show a positive correlation with the degree of hemolysis in SCD patients [65], while elevated IL-33 has also been observed to correlate with disease activity in autoimmune hemolytic anemia [66]. Given the ability of IL-33 to induce the secretion of pro-inflammatory cytokines, and promote responses by cytotoxic NK cells and regulatory T cell subsets [67], this red cell DAMP may conceivably mediate some of the significant inflammatory responses that are observed following hemolysis.

Mitchondrial (mt) DNA may have a role in sterile inflammation due to its ability to activate TLR9 and directly induce NLRP3 inflammasome formation [68, 69]. Furthermore, the inflammatory effects of extracellular mtDNA may be enhanced by the oxidative modifications that occur during oxidative stress [70]. While mature erythrocytes do not have functional mitochondria, they appear to retain some residual mtDNA, which has been detected in red cell products [71]. Cyclophilin A (CypA) is a DAMP that has been associated with rheumatoid arthritis, liver injury and severe sepsis and that can act as a chemotactic agent for inflammatory cells by binding to the CD147 receptor and directly stimulating the release of a number of inflammatory mediators [72–75]. Although, to our knowledge, extracellular CypA has not been associated with hemolysis, it has been identified in the erythrocyte cytosolic fraction by proteome studies [76], where it is suggested to persist from the reticulocyte stage of red cell maturation [77] and could contribute to the red blood cell DAMPs that are released upon erythrocyte lysis.

Inflammatory pathways stimulated by red cell DAMPs

Extracellular trap formation

Extracellular traps (ETs) are now thought to be essential for immune responses; these traps are formed by reticulate structures of extracellular DNA and granular proteins that can be released from monocytes, eosinophils and mast cells, but particularly from neutrophils [78]. Within minutes of activation by molecules such as lipopolysaccharide (LPS; the major component of the outer membrane of Gram-negative bacteria), neutrophils undergo a series of processes that include delobulation of the nucleus, chromatin condensation, disintegration of granular membranes and mixing of the cell's nuclear and cytoplasmic contents [79, 80]. These cellular contents are then ejected through the ruptured cell membrane, resulting in the extrusion of NETs that are more than tenfold the volume of the neutrophil. These NETs are thought to be important for host defense against infection to immobilize microorganisms and facilitate pathogen elimination [79, 81]; however, growing evidence indicates a role for NETs in a number of inflammatory and autoimmune diseases such as atherosclerosis and systemic lupus erythematosus [82, 83]. The presence of NETs has also been reported in SCD and transfusion-related acute lung injury (TRALI), a major cause of transfusion-related mortality [84, 85], both of which are characterized by vascular inflammation with implications for the participation of hemolytic processes or products (derived from stored blood products), respectively, in their pathophysiology [86–89].

The activation of neutrophils by heme to induce the production of inflammatory molecules, such as IL-8, ROS and cell migration, has been recognized for some time [36, 89, 90]. A crucial role for heme in inducing NET formation in neutrophils after proinflammatory cytokine priming has been suggested [91], where this NET production occurs in a ROS-dependent manner, and may be dependent on heme iron [88, 91]. Furthermore, there is evidence to indicate that NET formation is augmented in SCD, where they are likely pathogenic [84, 91]. Free heme has also been shown to induce the formation of mast cell extracellular traps (METs) in TNF-primed mice mast cells from SCD mice, possibly mediated by the action of TLR4, suggesting a contribution of heme-induced MET formation to SCD pathophysiology [92].

Red cell DAMPs and TLR pathways

TLRs are members of the superfamily of pattern recognition receptors (PRRs). TLRs are located on the cell surface

of both immune cells (e.g., macrophages and dendritic cells) and nonimmune cells (e.g., epithelial, fibroblast, and endothelial cells), where they can detect pathogen-associated molecular pattern (PAMPs) and endogenous inflammatory DAMPs [93, 94]. The TLRs are type I transmembrane glycoprotein receptors that contain leucine-rich repeat (LRR) domains for the recognition of PAMPs or DAMPs, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that mediates downstream signaling [95]. TLRs can be divided into two groups based on their cellular localization of ligand interaction: TLRs 1, 2, 4–6 and 11 are found on the cell surface, where they detect lipids, lipoproteins and proteins. In contrast, TLRs 3 and 7–9 are localized in the membranes of endosomal compartments (intracellular TLRs) and recognize bacteria- and virus-derived nucleic acids [96]. TLR expression differs on different cell types and, on the same cell type, signaling by different TLRs may result in diverse effects [97]. The pathways of cell activation and pathogen recognition in neutrophils, for example, are regulated by different TLRs that control oxidative mechanisms, inflammatory cytokine production and different cell death pathways, to control the cell's microbicidal and inflammatory activity [97, 98].

Of the potential red cell DAMPs, Hsp70 and mtDNA are both known to stimulate TLR (TLR2 and 4, and TLR9, respectively), while heme exerts a number of inflammatory effects via activation of TLR4 [38, 99, 100]. Methemoglobin (the precursor of heme) [101] causes dose-dependent, TLR4-dependent secretion of TNF from microglia and macrophages. Heme can also induce programmed necrosis in macrophages, by means of autocrine TNF and ROS production via a TLR4/MyD88-dependent pathway [102], and TLR4-mediated cerebral inflammation in an intracerebral hemorrhage (ICH) model [100, 103]. TNF, in turn, is a potent inflammatory cytokine that induces endothelial activation, lipid mediator expression and the activation of leukocytes [104], as well as modulating cell survival, differentiation and proliferation via the receptors TNF receptor 1 (TNFR1) and TNFR2. TNF has consistently been reported to be elevated in SCD and malaria [105–108], where it is assumed to play a major role in the inflammatory state associated with these diseases.

In the specific context of SCD, heme is reported to induce TLR4 activity, leading to acute lung injury in a sickle mice model [109] and induces vaso-occlusive-like processes via TLR4-mediated damage to endothelial cells in SCD mice [33]. Transcriptome profile hierarchical clustering identified TLR4 as being over 100-fold more expressed in the mononuclear cells of a group of SCD patient, compared to healthy controls [110], while the mast cells of SCD mice express significantly higher TLR4, as compared to control mice [111]. Individuals with malaria, another disease characterized by hemolysis [112], present elevated plasma heme concentrations, in association with a decrease in endothelial progenitor cells (EPC) and augmented TLR4 expression. In vitro experiments demonstrated that heme induced CXCL10 expression and apoptosis in human brain microvascular endothelial cells (HBVEC) and $CD34^{+}$ hematopoietic stem and progenitor cells (HSPC) via a TLR4-dependent pathway, indicating a role for the heme/TLR4 pathway in malaria pathogenesis [113].

Activation of TLRs on leukocytes, macrophages, other immune cells, vascular smooth muscle cells and endothelial cells can result in the increased production of proinflammatory cytokines and adhesion molecules, exacerbating the vascular inflammatory mechanisms that contribute to the pathophysiology of many of the hemolytic diseases, such as SCD and potentially causing damage to organs such as the kidneys and heart [96, 114]. Furthermore, TLRs have been implicated in pain signaling [115, 116], a finding that could be of relevance to SCD [117].

The inflammasome pathway and red cell DAMPs

The existence of the inflammasome was first proposed in 2002, when the association of caspase-1 activation and the maturation and release of IL-1β was first reported [118]. It is now well accepted that this mechanism is largely responsible for the maturation and release of the IL-1β and IL-18 cytokines, although a complex organization of multiple proteins and pathways is necessary for this to occur. In addition to cytokine maturation, the formation of the inflammasome results in cell pyroptosis, a type of programmed cell death [94]. IL-1β and IL-18 activate and stimulate adhesive interactions on endothelial cells, vascular smooth muscle cells and leukocytes via their interactions with the IL-1 type 1 receptor (IL-1RI) and the IL-18 receptor α (IL-18Rα), respectively. These cytokines also stimulate the release of IL-6 and IL-17 (IL-1β) and interferon(IFN)-γ, IL-2 and IL-12 (IL-18), which in turn stimulate T-helper (Th) 1 and Th17 type immune responses [119].

Inflammasome formation can occur in response to both microbial activators and sterile inflammatory signals, such as those released by damaged cells, and play a central role in controlling the host innate immune defense, the principal role of which is to maintain homeostatic tissue function [120]. The formation of inflammasomes in inflammatory cells, including macrophages, neutrophils and dendritic inflammatory cells, has been observed in a number of inflammatory diseases, including autoinflammatory and autoimmune diseases such as gout, diabetes type 2, Alzheimer's disease, atherosclerosis and obesity, and

evidence for a role of red cell DAMPs in inflammasome formation is growing. The inflammasome is formed by a multiprotein complex, containing PRRs, often represented by the NLRPs (also known as NOD-like receptors, NLRs), which recognize PAMPs or DAMPs. Once activated, the NLRs recruit multiple copies of the adapter protein, apoptosis-related speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1. Cleavage of pro-caspase-1 into its activated form then occurs, which in turn cleaves pro-interleukin IL-1β and pro-IL-18 into their bioactive forms [120–122].

Inflammasome components

Of the inflammasomes, the best characterized is the NLRP3 inflammasome, which is formed principally in neutrophils, macrophages, monocytes and dendritic cells in a rapid response to inflammatory stimuli [120]. In addition to the NLRP3 inflammasome, NLRP1, retinoic acid-inducible gene 1 (RIG-1), absent in melanoma (2AIM2) and IL-1β-converting enzyme protease-activating factor (IPAF) inflammasomes have also been reported [123]. Thus far, 22 NLRs have been identified in humans, and 34 in mice. The proteins of this family all present C-terminal leucine-rich repeats followed by a nucleotide-binding NACHT domain, but differ in their N-terminal effector domains. NLRP3 has a pyrin-like domain that is necessary for the recruitment of the adaptor molecule ASC [123, 124]. NLRP3 can recognize a wide variety of exogenous and endogenous stimuli such as microbial agonists, ATP, heme and mtDNA [34, 54, 69, 125]; however, the activation of NLRP3 by its DAMPs appears to occur via indirect mechanisms triggered by signaling intermediates, such as a reduction in intracellular potassium levels [126] and the generation of mitochondrial ROS [127, 128]. Following danger signal recognition, the activated NLRP3 rapidly induces polymerization of the ASC adapter protein into large helical filaments via pyrin interactions, which in turn provides a scaffold for the CARD-domain dependent recruitment and activation of pro-caspase-1 to form caspase 1 that subsequently induces the maturation and subsequent release of cytokines of the IL-1 family [129].

Inflammasome priming

In addition to the activation of inflammasome following the recognition of a PAMP/DAMP, a priming signal is also required for the production of active IL-1β to occur. This priming signal, or "signal 1", upregulates the gene transcription of the inflammasome machinery and the pro-form of IL-1β, which is then fed into the inflammasome for cleavage by caspase-1 to form active IL-1β [123]. For example, for heme to induce NLRP3 inflammasome formation in macrophages, these cells require priming by LPS to activate NF-κB and induce the expression of NLRP3, caspase-1 and IL-1β, probably via TLR-mediated signaling [34]. Additionally, NETs and LL-37 (a protein contained in NETs) can activate caspase-1, in human and murine macrophages [130, 131]. As such, cross talk between inflammatory pathways can occur to amplify inflammatory responses.

Heme, ATP and the inflammasome

Heme has been shown to induce IL-1β processing in mice bone marrow macrophages via the activation of the NLRP3 inflammasome, where inflammasome components were shown to contribute to hemolysis-induced lethality [34]. Heme induces Syk activation through its coordinated iron, via an unknown receptor, in turn inducing mtROS generation and NLRP3 activation. mtROS generation, NADPH oxidase 2 (NOX2) and K+ efflux are all essential for NLRP3 activation in this setting [34]. In another study, heme was suggested to activate NLRP3 inflammasome formation through P2X receptors, especially the P2X7R and P2X4R, with a potential role in kidney inflammation [132]. Increased extracellular ATP levels are also thought to amplify inflammatory responses by promoting NALP3-inflammasome assembly via the P2X7 receptor [54, 133].

Hemoglobin/heme scavenging proteins

The organism can counter hemoglobin release from red blood cells via scavenger proteins such as haptoglobin (HP) and hemopexin. HP, produced mainly by hepatocytes, is an acute phase plasma glycoprotein whose synthesis can be upregulated by inflammatory stimuli, particularly IL-6 [134]. The HP protein can bind tightly to free hemoglobin thereby preventing its oxidative effects and the extravasation of free hemoglobin into tissues. The hemoglobin/HP complex is then taken up by the liver and other tissues via receptors such as the CD163 receptor, found on macrophages and Kupffer cells [25, 135]. The human HP gene is polymorphic, resulting in three major HP genotypes, Hp1-1, Hp2-2 and Hp2-1, whose frequencies appear to vary according to geographical region. HP phenotype may have a significant influence on inflammatory status, particularly in inflammatory diseases where poor disease outcome has been consistently linked to the Hp2-2 phenotype [134, 136, 137], which while apparently preserving HP's hemoglobin-binding capacity [138, 139] may affect the clearance of the hemoglobin-HP complex and CD163-dependent anti-inflammatory signaling [140–142]. Current data regarding the influence of the HP genotype on the inflammatory status of the hemolytic diseases are scarce; the Hp2-2 genotype may

represent a risk for premature atherosclerosis and iron overload in children with transfusion-dependent β-thalassemia [143]; however, no HP genotypic effect on inflammatory cytokine production was reported in a population of individuals with SCA in Brazil [144]. Paradoxically, in malaria, some studies have reported data to suggest a protective effect of the HP2-2 phenotype against Plasmodium falciparum infection and inflammatory mediator production [145–148].

Hemopexin is another acute-phase plasma glycoprotein that is also produced principally by the liver in response to inflammatory stimuli [149]. Hempexin can bind heme (once released from oxidized hemoglobin), sequestering it in an inert form for transport to the liver [25] and preventing its pro-oxidant and and pro-inflammatory effects. Additionally, the small lipocalin protein, Alpha-1-microglobulin (A1M), found in plasma and tissues can also bind to and scavenge heme [150, 151]. The heme transported to the liver by hemopexin can then be cleaved by the HO-1 enzyme, generating biliverdin, carbon monoxide and iron. The expression of this enzyme is upregulated by hemoglobin/heme and represents a protective feedback mechanism [152, 153]. However, in events characterized by excessive intravascular hemolysis, these efficient hemoglobin/heme clearance mechanisms can be overwhelmed; indeed, HP and hemopexin depletion occurs in sickle cell anemia and other hemolytic anemias, and levels of these proteins may correlate inversely with disease severity [154–157].

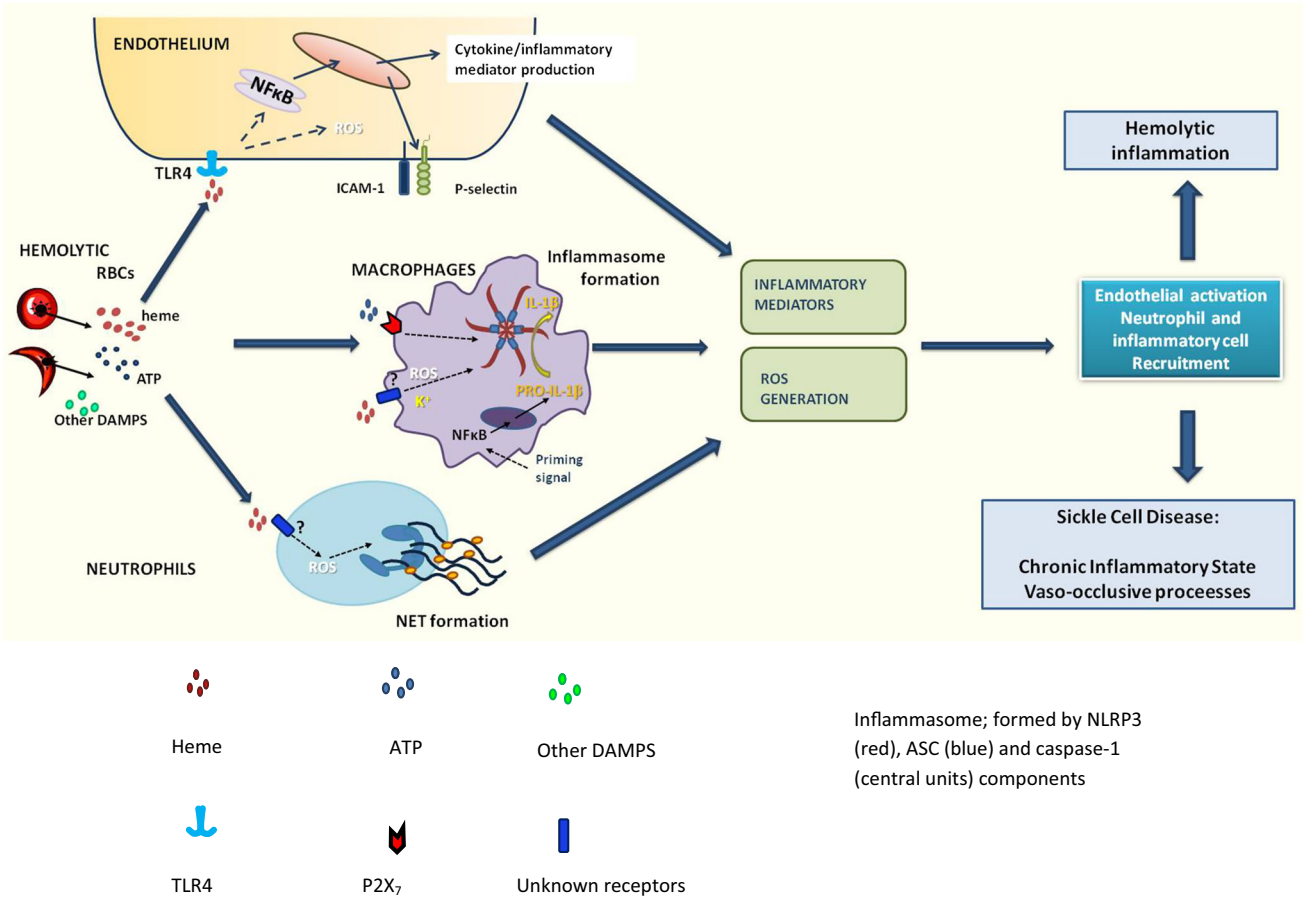

Fig. 2 Red cell DAMPs and inflammatory pathways. Damage-associated molecular patterns (DAMPs) are released from red blood cells during hemolysis. In turn, DAMPs such as heme, ATP and hsp70 activate endothelial cells, macrophages and leukocytes via interactions with membrane receptors such as toll-like receptor 4 (TLR4) or $P2X_7$, respectively, or other unknown receptors. In the endothelium, heme-mediated TLR4 signaling leads to reactive oxygen species (ROS) production and activates the NFκB transcription factor, resulting in the upregulation of surface adhesion molecules and inflammatory mediator production. Macrophages are previously primed, by lipopolysaccharide or cytokines such as TNF (or possibly by the binding of heme to TLR4), to produce Pro-IL-1β and other inflammasome components. Subsequent DAMP interaction induces the assembly of inflammasomes formed by the NLRP3 pattern recognition receptor, ASC and caspase-1. Pro-IL-1β is then cleaved by caspase-1 to form IL-1β. In neutrophils, heme can activate and promote NET formation via ROS generation. Subsequent inflammatory mediator production and ROS generation lead to further endothelial activation and leukocyte recruitment in the blood vessel, with ensuing amplification of inflammatory molecule release and, under certain circumstances, triggering of vaso-occlusive processes. Adapted from [18], with permission of Springer

Conclusion

Although hemolytic processes have long been associated with a decrease in red cell mass and, therefore, insufficient oxygen delivery to peripheral tissues [2], the major inflammatory consequences of intravascular hemolysis and their potential pathophysiological significance have only been recognized more recently. The release of large quantities of red cell DAMPs into the circulation, if not neutralized by innate protective mechanisms, has the potential to activate multiple inflammatory pathways that are known to participate in major inflammatory diseases. Indeed, evidence suggests that the major red cell DAMP, heme, is able to activate converging inflammatory pathways, such as TLR signaling, NET formation and inflammasome formation (Fig. 2), suggesting that this DAMP both activates and amplifies inflammation. As such, hemolysis represents a major inflammatory mechanism that potentially contributes to the clinical manifestations that have been associated with the hemolytic diseases, such as priapism, pulmonary hypertension and leg ulcers [16], and may contribute to specific complications of sickle cell disease such as endothelial activation, vaso-occlusive processes and tissue/organ injury.

Recognition of the huge inflammatory burden of hemolytic processes will pave the way for new approaches for use in combination with already established therapies aimed at treating specific aspects of the hemolytic diseases, with a view to either diminishing or neutralizing the effects of the release of hemoglobin and other DAMPs during red cell destruction. In sickle cell disease, for example, the prime approach for reducing the effects of hemolysis would be the use of gene editing or anti-hemoglobin-S-polymerizing therapies to prevent red cell lysis. However, in lieu of such therapies (or the only partial success of existing therapies), manners in which to neutralize hemoglobin and its products and the inflammatory downstream effects of red cell DAMP release in the circulation should be developed or optimized. Such approaches may include the use of purified or recombinant haptoglobin or hemopexin, NO donors (or modulators of downstream NO signaling, such as guanylate cyclase activators/inhibitors), anti-inflammatory drugs, such as anti-TNF agents or IL-1-receptor antagonists, and antioxidants.

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Compliance with ethical standards

Conflict of interest N. C. has received research funding from Bayer Pharma AG. The authors have no other potential conflicts of interests to declare.

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