Chapter 9
Pro-Inflammatory Actions of Red Blood Cell-Derived DAMPs

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Contents

Abstract Damage-associated molecular patterns (DAMPs) or alarmins are endogenous danger signals that are derived from damaged cells and extracellular matrix degradation, capable of triggering innate immune response to promote tissue damage repair. Hemolytic or hemorrhagic episodes are often associated with inflammation, even when infectious agents are absent, suggesting that damaged red blood cells (RBCs) release DAMPs.

Hemoglobin (Hb) composes 96% of the dry weight of RBCs; therefore upon hemolysis, tremendous amounts of Hb are released into the extracellular milieu. Hb oxidation occurs outside the protective environment of RBCs, leading to the formation of different Hb oxidation products and heme. Heme acts as a prototypic DAMP participating in toll-like receptor as well as intracellular nucleotide-binding oligomerization domain-like receptor signaling. Oxidized Hb forms also possess some

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inflammatory actions independently of their heme releasing capability. Non-Hb-derived DAMPs such as ATP, interleukin-33, heat shock protein 70, as well as RBC membrane-derived microparticles might also contribute to the innate immune response triggered by hemolysis/hemorrhage.

In this chapter we will discuss the inflammatory properties of RBC-derived DAMPs with a particular focus on Hb derivatives, as well as therapeutic potential of the endogenous Hb and heme-binding proteins haptoglobin and hemopexin in the prevention of hemolysis/hemorrhage-associated inflammation.

Keywords Hemoglobin · Red blood cells · Inflammasome · DAMPs · Hemolysis · Hemorrhage

Abbreviations

ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	Adenosine triphosphate
CO	Carbon monoxide
Cys	Cysteine
DAMPs	Damage-associated molecular patterns
FerrylHb	Ferrylhemoglobin
Hb	Hemoglobin
HO-1	Heme oxygenase-1
H_2O_2	Hydrogen-peroxide
Hp	Haptoglobin
Hsp	Heat shock protein
Hx	Hemopexin
ICAM-1	Intracellular adhesion molecule-1
ICH	Intracerebral hemorrhage
IL	Interleukin
LPS	Lipopolysaccharide
MetHb	Met(ferric) hemoglobin
Mhem macrophage	Hemorrhage-associated macrophage
MPs	Microparticles
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NRF2	Nuclear factor erythroid 2-related factor 2
PAMPs	Pathogen-associated molecular patterns
PPIX	Protoporphyrin IX

RBC	red blood cell
P2X7	P2X purinoceptor 7
TLR	Toll-like receptor
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tyr	Tyrosine
VCAM-1	Vascular cell adhesion molecule-1

9.1 Introduction

Damage-associated molecular patterns (DAMPs) or alarmins are endogenous danger signals that are derived from damaged cells and extracellular matrix degradation capable of triggering and/or exacerbating innate immune responses to promote tissue damage repair (Matzinger 1994). Hemolytic or hemorrhagic episodes are often associated with inflammation even when infectious agents are absent (Arruda et al. 2005), suggesting that damaged red blood cells (RBCs) release DAMPs (Mendonca et al. 2016).

The far most abundant protein in mature RBCs is hemoglobin (Hb) that composes 96% of the dry weight of RBCs; therefore upon hemolysis, tremendous amounts of Hb are released into the extracellular milieu. Once outside the protective environment of RBCs, Hb is prone to oxidation, in which process different Hb oxidation products form with diverse biological activities toward immune and nonimmune cells. Heme, the prosthetic group of Hb, is promptly released from oxidized Hb species and is the most studied RBC-derived alarmin (Soares and Bozza 2016). Heme is a strong prooxidant and is involved in toll-like receptor (TLR) as well as intracellular nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) signaling [reviewed in Dutra and Bozza (2014), Soares and Bozza (2016)]. Besides heme, oxidized Hb forms also possess some inflammatory actions independently of their heme releasing capability [reviewed in Jeney et al. (2014)]. Non-Hb-derived DAMPs such as adenosine triphosphate (ATP), interleukin (IL)-33, heat shock protein (Hsp) 70, as well as RBC membrane-derived microparticles (MPs) might also contribute to the innate immune response triggered by hemolysis/hemorrhage.

Deleterious effects of extracellular Hb and heme are controlled by haptoglobin (Hp) and hemopexin (Hx), respectively. These acute phase proteins bind extracellular Hb and heme avidly and facilitate their removal from circulation through receptor-mediated endocytotic routes. Upon massive intravascular hemolysis, the scavenging capacities of Hp and Hx are overwhelmed. Along with this notion, Hp- and Hx-based therapeutic interventions could be beneficial in pathologies associated with hemolysis/hemorrhage.

9.1.1 Physiology of RBCs

RBCs are the most prevalent cells in the human body, structurally and functionally dedicated to transport oxygen and carbon dioxide throughout the organism. RBCs are formed in the bone marrow from pluripotent hematopoietic stem cells in the process of erythropoiesis. Differentiation takes place mainly in the bone marrow, until reticulocytes released into the bloodstream where they mature further 1–2 days into terminally differentiated RBCs. During differentiation RBCs loose nuclei and cytoplasmic organelles including mitochondria and ribosomes. The advantage of not having nuclei in mature RBCs is twofold: first, anucleated cells are more flexible assuring that they can squeeze through small blood capillaries; second, there is more space for Hb resulting in increased oxygen-binding capacity. On the other side of this trade-off, mature anucleated RBCs are unable to divide, and their rescuing mechanisms are limited. This explains the relatively short life-span (100–120 days) of RBCs in the circulation, and the enormous turnover of making and breaking RBCs (200 billion RBCs/day).

Circulating RBCs are continuously exposed to high levels of reactive oxygen species (ROS) of both endogenous and exogenous origin [reviewed in Mohanty et al. (2014)]. Each ml of blood contains 0.3 g of Hb, and auto-oxidation (Table 9.1, equation #1) of Hb is the major source of endogenous ROS in RBCs. Besides Hb auto-oxidation, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases also contribute to endogenous ROS production in RBCs (George et al. 2013). To cope with this challenge, RBCs are equipped with a highly effective antioxidant defense system which includes enzymes such as Cu/Zn superoxide dismutase that convert superoxide anion to hydrogen peroxide (H_2O_2), catalase, glutathione peroxidase, and peroxiredoxins which decompose H_2O_2 to H_2O [reviewed in Siems et al. (2000), Jeney et al. (2013), Mohanty et al. (2014)]. Nonenzymatic low-molecular-weight scavengers such as glutathione and ascorbic acid also contribute to this

Table 9.1 Oxidative modifications of hemoglobin

	Formed species
(1) $Hb(Fe^{2+})O_2 \rightarrow Hb(Fe^{3+}) + O_2^{\bullet -}$	Methemoglobin
(2) $Hb(Fe^{2+})O_2 + H_2O_2 \rightarrow Hb(Fe^{4+} = O^{2-}) + H_2O + O_2$	Ferrylhemoglobin
(3) $Hb(Fe^{3+}) + H_2O_2 \rightarrow Hb^{\bullet +}(Fe^{4+} = O^{2-}) + H_2O$	Ferrylhemoglobin globin radical
(4) $Hb(Fe^{4+} = O^{2-}) + 2H^+ \rightarrow Hb^{\bullet +}(Fe^{3+}) + H_2O$	Methemoglobin globin radical
(5) $Hb^{\bullet +}(Fe^{3+}) + Hb^{\bullet +}(Fe^{3+}) \rightarrow (Fe^{3+})\ ^{+}Hb{-}Hb^{+}(Fe^{3+})$	Covalently cross-linked methemoglobin multimer

Routes of hemoglobin oxidation. Auto-oxidation of Hb generates metHb and superoxide anions (equation 1). H_2O_2 triggers a two-electron oxidation of Hb leading to the formation of ferryl ($Fe^{4+} = O^{2-}$) Hb (equation 2). The reaction of metHb with H_2O_2 yields ferrylHb radical ($Hb^{\bullet +}(Fe^{4+} = O^{2-})$) in which the unpaired electron is associated with the globin or the porphyrin ring (equation 3). FerrylHb can trigger further production of globin radicals via an intramolecular electron transfer between the ferryl iron and specific amino acid residues of the globin chains resulting in the formation of metHb globin radical (equation 4). Termination reactions of globin- and porphyrin-centered radicals lead to the formation of globin-globin (equation 5) cross-links

protection. Incomplete neutralization of ROS triggers RBC membrane damage and subsequent impairment of oxygen delivery to the tissues which eventually leads to tissue damage and inflammation.

Circulating RBCs lose 20% of their Hb content during their life-span via vesiculation (Willekens et al. 2003). Vesiculation is considered to be a self-protective mechanism of RBCs via which RBCs release membrane patches containing removal molecules including phosphatidylserine, immunoglobulin G, and senescent cell antigens (Willekens et al. 2008). Additionally, RBCs are able to get rid of intracellular inclusions, e.g., Heinz bodies via this mechanism (de Back et al. 2014), thereby postponing the premature loss of otherwise healthy RBCs from the circulation. RBC-derived vesicles are rapidly removed from the circulation by the mononuclear phagocyte system (Willekens et al. 2003).

At the end of their life-span, senescent RBCs are removed from the circulation by hemophagocytic macrophages, mainly in the spleen (Bratosin et al. 1998; de Back et al. 2014). Aged RBCs are smaller and denser because of the permanent loss of Hb and cell membrane via vesiculation and also characterized by decreased metabolic activity (Piomelli and Seaman 1993). At the terminal stage of RBC aging, "eat me" signals appear, and "don't eat me" signals disappear on the surface of senescent RBCs, and shortly after they are internalized by macrophages [reviewed in de Back et al. (2014)].

Different theories exist about the entity of the removal surface markers of senescent RBCs [reviewed in de Back et al. (2014)]. Phosphatidylserine, a phospholipid normally found in the inner membrane of RBCs, is a very likely candidate of being a removal signal, when it appears in the outer membrane of the RBCs (Boas et al. 1998). Phosphatidylserine is a general marker for apoptotic cells (Fernandez-Boyanapalli et al. 2009), and although RBCs cannot undergo a classical apoptosis because of the lack of nucleus and other cellular organelles, evidence suggest that aged or damaged RBCs can undergo a regulated process called eryptosis that is in many terms resembles to that of programmed cell death (Lang et al. 2005). Eryptosis is characterized by cell shrinkage, membrane blebbing, activation of proteases, and exposition of phosphatidylserine at the outer membrane leaflet of RBCs. Importantly, the removal of these phosphatidylserine-positive senescent, or terminally damaged RBCs by macrophages, is a non-inflammatory process and allows efficient and safe recycling of the RBC components, particularly the heme iron (Muckenthaler et al. 2017).

9.1.2 Hemolysis and Hemorrhage

Numerous pathologies are associated with hemolysis or hemorrhage characterized by uncontrolled destruction of RBCs. Hemolysis can occur in the vasculature but also in the extravascular space. Inherited or acquired conditions can cause hemolysis as listed in Table 9.2. Inherited hemolytic diseases are caused by mutations in genes encoding Hb, RBC membrane components, or certain enzymes in RBCs. The repertoire of acquired conditions associated with hemolysis is quite wide.

Table 9.2 Causes of hemolysis

Type	Cause of hemolysis	Example	Inflammasome activation
Inherited	RBC membrane abnormalities	Spherocytosis	Not reported
		Elliptocytosis	Not reported
	RBC metabolism abnormalities	G6PD deficiency	Not reported
		PK deficiency	Not reported
	Hemoglobinopathies	Thalassemias	Not reported
		Sickle cell disease	Yes (Cerqueira et al. 2011)
Acquired	Immune mediated (Autoimmune)	Warm antibody	Not reported
		Cold antibody	Not reported
	Immune mediated (Alloimmune)	Transfusion reaction	Controversial (Gibb et al. 2016, Land 2013)
		Hemolytic disease of the newborn	Not reported
	Mechanical, physical or chemical trauma	Microangiopathies	Not reported
		Prosthetic heart valves	Not reported
		Burns	Yes (Stanojcic et al. 2014)
		Heavy metal toxicity	Not reported
		Drug induced	Not reported
	Infections	Malaria	Controversial (Dostert et al. 2009, Reimer et al. 2010)

Auto- and alloimmune reactions, mechanical, physical, or chemical stress, and diverse infections can trigger substantial RBC lysis. RBCs outside the vasculature tend to lyse quickly; therefore hemorrhages are also associated with RBC lysis.

9.1.3 The Fate of Extracellular Hemoglobin

Hb is released in large amounts from lysing RBCs. Extracellular Hb exerts diverse unfavorable vasoactive effects. For example, extracellular Hb scavenges nitric oxide, an important vasodilator and signaling molecule in the vasculature [reviewed in Rother et al. (2005)]. Furthermore, once outside the protective environment of RBCs, Hb tends to oxidize. Auto-oxidation of Hb occurs resulting in metHb generation meanwhile superoxide anions are formed (Table 9.1, equation 1). Peroxides, such as H_2O_2 or lipid hydroperoxides, induce a two-electron oxidation of Hb leading to the formation of ferryl ($Fe^{4+} = O^{2-}$) Hb (Table 9.1, equation 2), whereas the reaction of metHb with H_2O_2 results in ferrylHb radical ($Hb^{\bullet+}(Fe^{4+} = O^{2-})$) in which the unpaired electron is located at either the globin chain or at the porphyrin ring (Table 9.1, equation 3) (Harel and Kanner 1988; Patel et al. 1996; Jia et al. 2007; Alayash et al. 2001). These high-valence iron compounds, i.e., ferrylHb and ferrylHb radical, are highly reactive intermediates that can decay by several ways

(Reeder et al. 2008). FerrylHb induces additional production of globin radicals via an intramolecular electron transfer between the ferryl iron and specific amino acid residues of the globin chains such as αTyr-24, αTyr-42, αHis-20, βTyr-35, βTyr-130, and βCys-93 leading to the formation of metHb globin radical (Table 9.1, equation 4) (Deterding et al. 2004; Ramirez et al. 2003; Jeney et al. 2013). Termination reactions of globin- and porphyrin-centered radicals lead to the formation of globin-globin (Table 9.1, equation 5) or porphyrin-globin crosslinks.

To prevent the deleterious effects of extracellular Hb, efficient mechanisms have evolved for its removal from the circulation. Hp, an acute-phase protein, is present in plasma in high amounts (0.41−1.65 mg/ml) with the special recognized function of capturing cell-free Hb [reviewed in Alayash (2011)]. The formation of the Hp-Hb complex is virtually irreversible, and Hp binding has multiple beneficial effects. First of all, Hp binding facilitates the removal of Hb from circulation through the CD163 macrophage scavenger receptor-mediated endocytosis (Kristiansen et al. 2001). Besides this effect, studies showed that Hb bound to Hp is less prone to H_2O_2-mediated oxidation than free Hb (Buehler et al. 2009; Banerjee et al. 2012; Miller et al. 1997). In fact, the Hb-Hp complex acts as a fairly efficient peroxidase (Kapralov et al. 2009). Further studies proved that Hp prevents H_2O_2-induced oxidation of amino acids in critical regions of Hb chains—i.e., α-Tyr42, β-Tyr145, and β-Cys93—and polymerization of Hb (Pimenova et al. 2010). The recent determination of the crystal structure of the porcine Hp-Hb complex revealed that Hb residues known to be prone to oxidative modifications are buried in the Hp-Hb interface, thereby explaining this direct protective role of Hp against H_2O_2-induced oxidation (Andersen et al. 2012).

Although the Hb/Hp/CD163 system is highly efficient in removing intravascular free Hb, it has some limitations. Plasma Hp can bind and clear approximately 3 g of Hb from the circulation which is less than 1% of the total amount of circulating Hb. In case of pronounced hemolysis, when more than 1% of RBCs disrupt, Hp is depleted from the circulation in which case free Hb is cleared (rather inefficiently) via a low-affinity pathway through CD163 (Schaer et al. 2006) and/or by renal excretion (Schaer et al. 2013; Murray et al. 1961). This latter is accompanied by generation of free iron and organ damage.

Another limitation of the Hp/CD163 system is that Hp and CD163 have decreased affinity for structurally altered (e.g., covalently cross-linked) Hb species that might form upon Hb oxidation. Recent studies have revealed that elimination of oxidized Hb species via both high-affinity and low-affinity pathways can be severely compromised (Schaer et al. 2006; Vallelian et al. 2008).

Upon massive hemolysis Hp is consumed, causing accumulation and oxidation of cell-free Hb that eventually lead to the release of the prosthetic heme group. Hx is an acute-phase plasma protein that binds heme with the highest affinity of any known heme-binding proteins (Hrkal et al. 1974). Hx-heme complexes are internalized via the scavenger receptor LDL receptor-related protein 1/CD91 (Hvidberg et al. 2005) mainly by hepatocytes and macrophages (Herz and Strickland 2001).

Following internalization of Hb or heme, cells and tissues upregulate heme oxygenase-1 (HO-1) and ferritin. HO-1 catabolizes free heme into equimolar amounts of Fe^{2+}, carbon monoxide (CO), and biliverdin (Tenhunen et al. 1968).

Liberated iron drives the upregulation of ferritin that is the main intracellular iron storage protein (Eisenstein et al. 1991).

9.1.4 Pro-inflammatory Actions of Hb-Derived Species

Massive intravascular hemolysis or hemorrhage result in the exhaustion of the endogenous defense system leading to the accumulation of oxidized Hb forms and free heme in the plasma or in the extravascular space (Pamplona et al. 2007; Larsen et al. 2010; Nagy et al. 2010). These Hb derivatives, particularly free heme, exert prooxidant activities [reviewed in Immenschuh et al. (2017), Jeney et al. (2013)]. Moreover, hemolytic or hemorrhagic episodes are often associated with inflammation even when infectious agents are absent (Arruda et al. 2005). Considerable effort has been made to define the mediators and the target cells involved in the hemolysis-/hemorrhage-induced inflammatory response. Accumulating evidence suggest that Hb-derived oxidized species possess diverse pro-inflammatory actions targeting different immune and non-immune cells (Table 9.3).

9.1.4.1 Macrophage Activation

Macrophages, the frontline cells of innate immunity, respond to a variety of pathogen-associated molecular patterns (PAMPs) and DAMPs. Lysis of RBCs leads to the release of different RBC components that can potentially behave as DAMPs and induce a sterile inflammatory response dependently of receptors such as TLRs or NOD-like receptors (Table 9.3).

Accumulating evidence suggests that heme that is released from oxidized Hb forms modulate macrophage phenotype. Bozza et al. showed that heme triggers tumor necrosis factor-alpha (TNF-α) secretion by macrophages in a TLR4-dependent manner (Figueiredo et al. 2007). The activation of TLR4 by heme is strictly dependent on its coordinated iron and the vinyl groups of the porphyrin ring (Figueiredo et al. 2007). Sustained exposure of macrophages to free heme triggers programmed necrosis that is dependent on autocrine production of TNF-α and ROS (Fortes et al. 2012). The pathogenic role of heme-mediated TLR4 activation was investigated in a murine model of intracerebral hemorrhage (ICH)-induced neuro-inflammation. In comparison to wild-type mice, $TLR4^{-/-}$ mice exhibited less inflammation, reduced cerebral edema, and lower neurological deficit scores, suggesting that heme-mediated TLR4 activation plays a critical role in ICH-associated neuro-inflammation (Lin et al. 2012). Gram et al. showed that after intraventricular hemorrhage, metHb forms and its level correlates to the expression of TNF-α (Gram et al. 2013). In agreement with this finding, a recent study of Kwon et al. revealed that metHb is an important endogenous activator of TLR4 that promotes widespread TLR4-mediated neuro-inflammation upon subarachnoid hemorrhage (Kwon et al. 2015).

Table 9.3 Pro-inflammatory actions of RBC-derived DAMPs

DAMP	Major finding	References
Heme	Heme triggers TLR4-dependent TNF-α secretion in macrophages	Figueiredo et al. (2007)
Heme	Heme-mediated activation of TLR4/MyD88/TRIF pathway plays a role in intracerebral hemorrhage	Lin et al. (2012)
MetHb	MetHb and TNF-α levels correlate in cerebrospinal fluid after intraventricular hemorrhage	Gram et al. (2013)
MetHb	MetHb promotes TLR4-dependent neuroinflammation upon subarachnoid hemorrhage	Kwon et al. (2015)
Heme	Heme induces NLRP3 activation and IL-1β secretion in LPS-primed macrophages	Dutra et al. (2014)
Heme	Heme triggers neutrophil recruitment, ROS production and IL-8 expression	Graca-Souza et al. (2002)
FerrylHb	FerrylHb triggers neutrophil recruitment in vivo independently of TLR4 activation	Silva et al. (2009)
Heme	Heme induces neutrophil extracellular trap formation	Chen et al. (2014)
Heme	Heme induces TLR4-dependent endothelial activation	Belcher et al. (2014)
FerrylHb	FerrylHb activates NF-κB, upregulates pro-inflammatory adhesion molecule expressions, and disrupts monolayer integrity in endothelial cells	Silva et al. (2009)
ATP	ATP activates P2X7 receptors leading to IL-1β secretion in LPS-primed macrophages	Perregaux et al. (2000)
ATP	ATP activates NF-κB, upregulates E-selectin expression, and induces deterioration of endothelial barrier function via acting on P2X7 receptors	McClenahan et al. (2009)
ATP	ATP induces NLRP3 activation and IL-1β secretion in LPS- or TNF-primed endothelial cells	Huck et al. (2015), Champaiboon et al. (2014)
ATP	ATP induces microparticle release, ROS formation, and apoptotic cell death in erythroid progenitor cells through activation of P2X7 receptors	Constantinescu et al. (2010), Wang and Sluyter (2013)
ATP	ATP triggers eicosanoid release, phosphatidylserine exposure, and lysis of mature RBCs through activation of P2X7 receptors	Jiang et al. (2006), Sluyter et al. (2007a, b)
HSP70	HSP70 activates macrophage IL-12 and E-selectin production in a TLR2/TLR4-dependent manner	Vabulas et al. (2002) Tsan and Gao (2004)
RBC MPs	RBC-derived MPs amplify thrombin-dependent activation of the complement system	Zecher et al. (2014)
RBC MPs	RBC-derived MPs enhance coagulation activation	van Beers et al. (2008)
RBC MPs	RBC-derived MPs activate endothelial cells via heme transfer	Camus et al. (2015)
RBC MPs	RBC-derived MPs are internalized by myeloid cells and induce pro-inflammatory cytokine production	Awojoodu et al. (2014)
RBC MPs	RBC-derived MPs contribute sickle cell disease-associated vascular dysfunction and cardiovascular complications	Tantawy et al. (2013b)
RBC MPs	RBC-derived MPs contribute to transfusion-induced inflammatory response	Cognasse et al. (2015)

Activation of the cytosolic NOD-like receptors results in the assembly of a caspase-1-activating scaffold. Active caspase-1 subsequently cleaves the pro-inflammatory IL-1 family of cytokines into their bioactive forms, IL-1β and IL-18, those can trigger pyroptosis, a type of inflammatory cell death [reviewed in Guo et al. (2015)]. The NLR family pyrin domain containing 3 (NLRP3) inflammasome, which belongs to the NOD-like receptor family, is the most extensively studied inflammasome, that is formed after the oligomerization of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and pro-caspase-1 (Schroder and Tschopp 2010).

Besides PAMPs, the NLRP3 inflammasome is activated in response to a wide variety of DAMPs including extracellular ATP, crystals of monosodium urate or cholesterol, β-amyloid fibers, the degradation of extracellular matrix components, and environmental or industrial particles and nanoparticles (Martinon et al. 2006; Mariathasan et al. 2006; Duewell et al. 2010; Halle et al. 2008; Babelova et al. 2009; Yazdi et al. 2010; Hornung et al. 2008).

Recently heme was added to the long list of NLRP3 activating danger signals. Dutra et al. showed that heme triggers active IL-1β production in lipopolysaccharide (LPS)-primed macrophages in an NLRP3- and caspase-1-dependent manner (Dutra et al. 2014). They also investigated the structural requirements of heme-mediated NLRP3 inflammasome activation. Heme analogs such as protoporphyrin IX (PPIX) that lacks the central iron atom or metal substitution derivatives such as CoPPIX and SnPPIX were unable to induce IL-1β secretion in LPS-primed macrophages (Dutra et al. 2014). Based on these observations, they came to the conclusion that NLRP3 activation by heme is strictly dependent on its coordinated iron, which is in conflict with the findings of Li et al. who reported that PPIX is as efficient in inducing IL-1β maturation and secretion as heme (Li et al. 2014).

9.1.4.2 Neutrophil Activation

Polymorphonuclear neutrophils are the first leukocytes migrating from the blood into injured or infected tissues. Neutrophils kill pathogens via various cytotoxic mechanisms and clear cellular debris; therefore they play a fundamental role in innate and adaptive immunity (Rosales et al. 2016). In the recent years, it has become evident that neutrophils not only sense PAMPs but can recognize and respond to endogenous DAMPs as well. In line of this notion, heme triggers neutrophil chemotaxis and activation, characterized by elevated ROS production and increased expression of the pro-inflammatory cytokine IL-8 (Graca-Souza et al. 2002). Heme-induced neutrophil recruitment is regulated through signaling pathways that are characteristic of chemoattractant molecules (Porto et al. 2007) but independent of TLR4-mediated signaling (Figueiredo et al. 2007). Besides heme, oxidized Hb (ferrylHb) is a very potent trigger of neutrophil infiltration in mice independently of TLR4 signaling (Silva et al. 2009). Additionally, Kono et al. showed that PPIX was as efficient as heme in inducing neutrophil ROS production, pointing out that this effect is independent of the coordinated iron present in heme (Kono et al. 2013).

Protoporphyrin ring-induced neutrophil activation was suggested to play a role in transfusion-related acute lung injury (Kono et al. 2013).

Additionally of ROS generation and the release of microbicidal molecules, neutrophils can release extracellular traps—a meshwork of chromatin fibers decorated by granular proteins—that represent an important strategy to immobilize and kill invading microorganisms (Brinkmann et al. 2004). Recently Chen et al. reported that heme is able to induce the formation of neutrophil extracellular traps and suggested that this mechanism contributes to vaso-occlusion crises in sickle cell disease (Chen et al. 2014).

9.1.4.3 Endothelial Cell Activation

Endothelium, the interface between blood and tissue, has a pivotal role in the inflammatory response mainly through the induction of the leukocyte adhesion cascade to facilitate transmigration of inflammatory cells to the inflamed tissue. Accordingly, inflammatory stimuli, such as IL-1, TNF-α, or LPS, upregulate cellular adhesion molecules including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E selectin, in endothelial cells (Bevilacqua et al. 1985; Pohlman et al. 1986). Wagener et al. found that exposure of endothelial cells to heme upregulated the expressions of ICAM-1, VCAM-1, and E selectin, in a similar manner to that of IL-1, TNF-α, or LPS (Wagener et al. 1997). Recently Belcher et al. showed that heme activates endothelial cells in a TLR4-dependent manner and that this heme-mediated TLR4-dependent endothelial activation plays a pathogenic role in vaso-occlusion in a murine model of sickle cell disease (Belcher et al. 2014).

While searching for other mediators of hemolysis-associated inflammation, Silva et al. reported that ferrylHb but not native Hb or metHb triggers upregulation of the pro-inflammatory adhesion molecules ICAM-1, VCAM-1, and E-selectin (Silva et al. 2009). FerrylHb induced rearrangement of actin cytoskeleton in endothelial cells leading to the disruption of the endothelial monolayer integrity (Silva et al. 2009). FerrylHb-induced inflammatory response was dependent on actin polymerization and the activation of the c-Jun N-terminal kinase and the p38 mitogen-activated protein kinase signal transduction pathways (Silva et al. 2009). Silva et al. showed that induction of endothelial inflammatory response is a unique property of ferrylHb because neither Hb nor metHb triggered these effects (Silva et al. 2009). FerrylHb can release its prosthetic heme group (Potor et al. 2013), and one can ask whether ferrylHb-mediated inflammatory response is mediated by the released heme. Many lines of evidence suggest that in fact this is not the case. First of all, metHb, that can also release heme in a similar manner as ferrylHb, does not induce inflammatory response in endothelial cells (Silva et al. 2009). Second, ferrylHb-induced inflammatory response is not dependent on TLR4 signaling (Silva et al. 2009). These results suggest that heme and ferrylHb are two Hb-derived pro-inflammatory agonists that trigger endothelial activation via different signaling mechanisms.

9.1.5 Cytoprotective and Anti-inflammatory Actions of Hb-Derived Species

Interestingly enough, besides its prooxidant and pro-inflammatory actions, under special circumstances heme can induce cytoprotective and anti-inflammatory responses. These protective mechanisms largely rely on the heme-mediated upregulation of the HO-1/ferritin system [reviewed in Gozzelino et al. (2010)], and it mostly relies on the ability of HO-1 to degrade heme into CO, iron, and biliverdin, in which the latter is promptly converted to bilirubin. The subsequent upregulation of ferritin is essential to obtain the protective effect, as it can store the released iron in a catalytically inactive form (Balla et al. 1992). Additionally, the side products of heme degradation, i.e., bilirubin and CO, exert diverse antioxidant and anti-inflammatory actions (Gozzelino et al. 2010).

Along with these notions, a subset of macrophages, called hemorrhage-associated or Mhem macrophages with anti-inflammatory properties, were identified in atherosclerotic plaques with intraplaque hemorrhage (Boyle et al. 2009). Mhem macrophages are characterized by facilitated iron sequestration assured by elevated expressions of HO-1 and CD163 and at the same time protection from foam cell formation secured by induction of genes central to cholesterol efflux (Boyle et al. 2009, 2012). Boyle et al. also showed that Mhem macrophage polarization is driven by heme and identified two key transcription factors nuclear factor erythroid 2-related factor 2 (NRF2) and activating transcription factor 1 involved in this process (Boyle et al. 2011, 2012).

Endothelial cells can also benefit from the cytoprotective mechanism provided by the HO-1/ferritin system. In the early 1990s, Balla et al. showed that a brief exposure of sublethal concentration of heme made endothelial cells highly resistant to subsequent oxidant-mediated killing in which cytoprotection was relied on the upregulation of the HO-1/ferritin system (Balla et al. 1992). Since that initial work, many investigations targeted the multifunctional role and therapeutic potential of HO-1 in the vascular endothelium [reviewed in Calay and Mason (2014)].

9.1.6 Non-Hb-Derived RBC DAMPs

Although Hb is the far more abundant molecule in RBCs, there are other components in RBCs that can potentially become DAMPs following RBC lysis. For example ATP, a universal energy source, is present in RBCs in high concentration (~1.6 mmol/L). When present in the extracellular milieu, ATP becomes a signaling molecule that activates P2 receptors in diverse cells (Dubyak 1991). It has been shown that hypoxia, elevated shear stress, and reduced pH lead to ATP release from RBCs, although it is still a matter of debate whether it occurs via an active or passive process. Bergfeld et al. showed that under hypoxic conditions, RBCs release ATP in a regulated way through the plasma membrane protein band 4.5 (Bergfeld and

Forrester 1992). Recently Sridharan et al. proposed that pannexin 1, a channel-forming glycoprotein, is involved in hypoxia-mediated ATP release from RBCs (Sridharan et al. 2010). Regarding shear stress-induced ATP release, Wan et al. suggested that mechanosensitive ATP release is triggered by retraction of the spectrin-actin cytoskeleton network and influenced by membrane viscosity (Wan et al. 2008). Recently, Piezo1, a mechanically activated cation channel involved in physiological responses to touch, pressure, and stretch, was shown to regulate mechanosensitive release of ATP from RBCs via controlling the shear-induced calcium influx (Cinar et al. 2015). Contrary to the active process, Sikora et al. reported that hemolysis is the primary mechanism via which RBCs release ATP in response to hypoxia or mechanical stress (Sikora et al. 2014). Nevertheless, RBC-derived ATP can activate P2 purinergic receptors on vascular endothelial cells, resulting in the synthesis of powerful vasodilators such as nitric oxide and prostaglandins (Burnstock 2017). Via this mechanism RBCs actively participate in the regulation of microvascular blood flow and contribute to match oxygen delivery and local needs (Ellsworth et al. 1995).

Besides its vasoactive effects, activation of P2 purinergic receptors by ATP can trigger inflammatory responses in various immune and nonimmune cells (Idzko et al. 2014). For example, ATP activates P2X purinoceptor 7 (P2X7) and promotes IL-1β and IL-18 secretion in LPS-primed macrophages (Perregaux et al. 2000). Activation of P2X7 receptors by ATP on endothelial cells leads to nuclear factor kappa B (NF-κB) activation and subsequent upregulation of its target genes such as E-selectin (von Albertini et al. 1998). Extracellular ATP induces deterioration of endothelial barrier function and may trigger apoptotic cell death (McClenahan et al. 2009). ATP can induce activation of the NLRP3 inflammasome and subsequent release of low levels of IL-1β in endothelial cells primed with LPS or TNF-α (Huck et al. 2015; Champaiboon et al. 2014). Furthermore, both progenitor and mature RBCs express P2 purinergic receptors, and accumulating evidence suggest that extracellular ATP exerts various biological effects on these cells (Burnstock 2015; Sluyter 2015). ATP induces the release of MPs, ROS formation and apoptotic cell death in erythroid progenitor cells (Chahwala and Cantley 1984; Constantinescu et al. 2010; Wang and Sluyter 2013). Activation of P2 purinergic receptors in mature RBCs triggers eicosanoid release and phosphatidylserine exposure and eventually leads to hemolysis (Jiang et al. 2006; Sluyter et al. 2007a, b).

IL-33, the member of the IL-1 cytokine superfamily, is a well-known alarmin that is released upon stress and contributes to the pathogenesis of diverse inflammatory diseases through the activation of innate immune cells (Rider et al. 2017). Recently Wei et al. showed that RBCs contain IL-33 and that IL-33 is released in large amounts upon RBC lysis (Wei et al. 2015). They found association between plasma IL-33 levels and the degree of hemolysis in sickle cell disease patients with intravascular hemolysis (Wei et al. 2015). Similar association between plasma IL-33 concentration and hemolysis was reported in patients with autoimmune hemolytic anemia (Bu et al. 2015). Released IL-33 signals through ST2 receptors and enhances the functions of diverse lymphoid and myeloid immune cells [reviewed in Griesenauer and Paczesny (2017)].

Hsps are ubiquitously expressed proteins exerting diverse protective mechanisms during cellular stress. For example, both constitutive and inducible forms of the 70 kDa Hsp, Hsc70, and Hsp70, respectively, function as cytosolic chaperons during erythrocyte maturation. Although the expressions of Hsc70 and Hsp70 decrease significantly at the terminal stage of erythroid progenitor cell differentiation (Patterson et al. 2009), they are still present in mature RBCs (Gromov and Celis 1991). Vabulas et al. showed that extracellular Hsp70 activates macrophage IL-12 and E-selectin production via CD14/TLR2 and CD14/TLR4 receptor complex-mediated signal transduction pathways (Vabulas et al. 2002). However, recent evidence suggests that the reported cytokine effects of Hsp70 and other Hsps may be due to the contaminating LPS (Tsan and Gao 2004).

Microparticles (MPs) are small membrane-encapsulated vesicles present in body fluids. Blood MPs can originate from platelets, RBCs, leukocytes, or endothelial cells. They are shed from cells in response to cell activation, cell stress, or apoptosis, and besides the phospholipid bilayer, they contain cytosolic components of their parental cells. RBCs release MPs during their normal lifetime in which process they lose a substantial amount of Hb content and surface area (Willekens et al. 2003). Hemoglobinopathies, characterized by shortened life-span of RBCs, such as sickle cell disease and thalassemia major, are associated with accelerated formation of RBC-derived MPs (Tantawy et al. 2013a, b). Interestingly increased levels of RBC-derived MPs are present in patients with metabolic syndrome (Helal et al. 2011). Recently RBC-derived MPs attracted attention in transfusion medicine as well. For therapeutic interventions, packed RBCs are stored in the blood bank for up to 42 days. Storage is associated with diverse morphological and biochemical alterations of RBCs including reduced integrity of the RBC membrane and the formation of RBC-derived MPs (Kim-Shapiro et al. 2011; D'Alessandro et al. 2015). RBC-derived MPs exert diverse biological actions. For example, RBC-derived MPs scavenge nitric oxide (Donadee et al. 2011; Liu et al. 2013) and amplify systemic inflammation via thrombin-dependent activation of complement system (Zecher et al. 2014). Moreover, RBC-derived MPs enhance coagulation activation (van Beers et al. 2008) and are involved in endothelial activation via heme transfer (Camus et al. 2015). RBC-derived MPs are internalized by myeloid cells and induce pro-inflammatory cytokine secretion (Awojoodu et al. 2014). These mechanisms contribute significantly to sickle cell disease-associated vascular dysfunction and cardiovascular complications (Tantawy et al. 2013b) and involved in transfusion-induced inflammatory responses (Cognasse et al. 2015).

9.1.7 Therapeutic Interventions

Different therapeutic approaches were designed and investigated to limit the pathological consequences of massive hemolysis or hemorrhages. Some strategies are focusing on limiting the formation or fostering the elimination of RBC-derived prooxidant and pro-inflammatory molecules. For example, Pamplona et al. showed

that CO—the product of heme catabolism—suppress the pathogenesis of experimental cerebral malaria. The effect is mediated by the binding of CO to Hb, preventing Hb oxidation and the generation of free heme, a molecule that plays a critical role in the pathogenesis of cerebral malaria (Pamplona et al. 2007). Recently the therapeutic potential of the natural plasma Hb and heme scavenger proteins, Hp and Hx, have been tested in preclinical animal studies and in small-scale human studies [reviewed in Schaer et al. (2013), Smith and McCulloh (2015)]. In humans Hp supplementation prevented hemoglobinuria or the development of acute kidney injury in a variety of hemolytic conditions [reviewed in Schaer et al. (2013)]. Vinchi et al. showed that Hx therapy improves cardiovascular function in mouse models of sickle cell anemia and β-thalassemia by preventing endothelial dysfunction (Vinchi et al. 2013) and inhibits heme-induced pro-inflammatory phenotypic change of macrophages in a mouse model of sickle cell disease (Vinchi et al. 2016).

Other therapeutic approaches against hemolysis-/hemorrhage-associated adverse effects rely on the induction of the natural antioxidant response. For example upregulation of the NRF2/HO-1 system suppresses the pathogenesis of severe malaria in mice, a pathology driven by RBC-derived heme (Pamplona et al. 2007; Ferreira et al. 2008; Seixas et al. 2009; Jeney et al. 2014). The protective mechanism provided by the NRF2/HO-1 system is very complex and relies on the effective removal of heme, the cytoprotective and anti-inflammatory actions of heme degradation products (bilirubin and CO), and the upregulation of the iron-sequestering protein, ferritin (Gozzelino et al. 2010).

9.2 Conclusions

The RBC is usually a blessing but sometimes a curse. It is a blessing, when it functions properly: circulates throughout the body about 170,000 times during its lifetime to deliver oxygen and remove carbon dioxide from cells and phagocytosed unperceivably at the end of its life-span by macrophages, and curse, when it is involved in pathophysiologic mischief upon hemorrhage or intravascular hemolysis.

Since the dogma breaking “danger model” introduced by Polly Matzinger in 1994 our understanding of how the immune system discriminates between dangerous and safe by recognition of pathogens or alarmins released by injured or stressed cells, underwent a fundamental revision. Diverse endogenous DAMPs were identified and their critical contributions were unquestionably verified in different pathologies. In the last decade, it became evident that upon hemolysis or hemorrhage RBCs release DAMPs that can activate immune and nonimmune cells via diverse signaling mechanisms. A lot of work needs to be done in the future to complete the colorful picture of RBC-derived DAMPs, their targeted cells, and the mechanisms of their actions. Fuller understanding of hemolysis/hemorrhage-associated inflammation could contribute to the development of novel therapeutics intended to interrupt these pathological events.

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