A Perspective on the Role of Extracellular Hemoglobin on the Innate Immune System

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Host cell-derived danger-associated molecular patterns (DAMPs), such as the hemoglobin (Hb) can interact with the innate immune system either directly or through binding to pathogen-associated molecular patterns (PAMPs). Hemolysis occurs under various pathological conditions, leading to hemoglobinemia. In the extracellular environment, the Hb becomes a redox-reactive DAMP molecule. In severe hemolysis, the massive level of extracellular pro-oxidative Hb generates reactive oxygen species (ROS), which perturbs the innate immune homeostasis. The Hb also binds to PAMPs and triggers Toll-like receptor-mediated signal transduction. In this perspective, we review the roles of cell-free Hb in the innate immune system, focusing on the plausible interactions among Hb, pathogens, host cell components, and innate immune cells, all of which remain to be explored with experiential detail.

The Roles of Reactive Oxygen and Nitrogen Species in Innate Immunity

THE IMPORTANCE OF reactive oxygen (ROS) and nitrogen (RNS) species in innate immune defense has been well recognized (Nathan and Ding, 2010; Wink et al., 2011). Although ROS exerts deleterious effects, such as mutagenesis and aging, it is indispensible for the maintenance of the innate immune system. The positive effects of ROS and RNS are displayed in chronic granulomatous disease (CGD), which is a rare inherited genetic disorder attributable to the lack of NADPH oxidase. The CGD individual is defective in producing superoxide anions and is prone to infection by peroxidase-positive pathogens (Hampton et al., 1998; Song et al., 2011). Under severe infection and chronic inflammation, CGD patients suffer enlarged lymph nodes, pulmonary fibrosis, and widespread tissue granulomas and Crohn-like colitis. IFNγ treatment-mediated nitric oxide (NO) production was reported to rescue the impaired phagocytosis of apoptotic cells in CGD patients (Fernandez-Boyanapalli et al., 2010; Naderi Beni et al., 2012); hence, the positive attribute of NO to innate immune defense.

In wild-type mice, lipopolysaccharide (LPS) induces p47phox-dependent ROS production and IL-10 secretion from the macrophage (Deng et al., 2012). On the other hand, when challenged with LPS, mice deficient in p47phox (a component of the NADPH oxidase), suffer severe lung inflammation due to the decreased anti-inflammatory cytokine, IL-10. NADPH oxidase-deficient mice also show increased susceptibility to infection by Listeria monocytogenes (Dinauer et al., 1997). The NO controls Th1 immune response

by regulating inflammatory-like DC subset activation (Giordano et al., 2011). Mice lacking inducible nitric oxide synthase (iNOS, which produces NO), are more susceptible to Leishmania major infection (Wei et al., 1995) and experimental autoimmune encephalomyelitis (Sahrbacher et al., 1998). Besides playing a crucial role in infection and immunity, other biologically beneficial functions of NO include neurotransmission, intracellular signaling, and regulation of tumorigenesis.

Hemoglobin-Mediated Oxidative Stress

Under tightly regulated conditions, ROS and RNS are key players in maintaining normal physiology. However, excessive levels of these pro-oxidative free radicals may overwhelm and impair our antioxidants, causing protein oxidation, lipid peroxidation, and nucleic acid oxidation, and leading to cellular dysfunction and cell death (Auten and Davis, 2009).

Hemoglobin (Hb) possesses pseudoperoxidase (POX) activity, which is triggered synergistically by microbial proteases and pathogen-associated molecular patterns (PAMPs), such as LPS and LTA, to produce superoxide anion (Jiang et al., 2007). Hb is an iron-containing metalloprotein normally sequestered in the erythrocytes to transport oxygen. However, under certain pathophysiological conditions, hemolysis occurs, releasing massive amounts of Hb into the blood stream. For instance, infection by bacteria, such as Staphylococcus aureus (Grenny and Stevens, 1935), parasites, such as Plasmodium falciparum (Rudzinska et al., 1965), and influenza virus (Maeda et al., 1981), may induce hemolysis. Hemolysis

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is also a common symptom in many inherited and acquired hematologic disorders, such as sickle cell anemia, glucose-6-phosphate dehydrogenase deficiency, and paroxysmal nocturnal hemoglobinuria. Transfusion with improperly stored red blood cells (Hod *et al.*, 2010) and treatment with anti-infective anti-inflammatory drugs, such as cotrimoxazole, ciprofloxacin, fludarabine, lorazepam, and diclofenac, may also induce hemolysis (Garbe *et al.*, 2011).

The released cell-free Hb scavenges NO, which normally plays an important beneficial role in vascular homeostasis, without which, smooth muscle dystonia, vasculopathy, thrombosis, endothelial dysfunction, and platelet aggregation may ensue (Reiter *et al.*, 2002; Olson *et al.*, 2004; Rother *et al.*, 2005). Peroxynitrite produced by the reaction between Hb and NO generates reactive hydroxyl radicals, which can initiate membrane lipid peroxidation and cellular damage (Auten and Davis, 2009). The plasma level of heme, which is increased by elevated proteolytic digestion of cell-free Hb in pathological conditions, such as cystic fibrosis, is also known to induce inflammation, coagulation, and tissue damage. In other instances, the highly expressed proteases in the lung of cystic fibrosis patient, cleave Hb to release heme and this increases the level of iron in the lung, which is exploited by pathogenic bacteria as nutrient. Generally, free heme is highly redox-reactive and induces oxidative stress. Thus, hemolysis unleashes Hb as a double-edged sword intraerythrocytic Hb, which normally transports oxygen for our survival, transforms into a redox-reactive danger-associated molecular pattern (DAMP) when it becomes extracellular and poses oxidative stress, turning the tide against our survival.

Toll-Like Receptors in Innate Immune System–Implications in ROS and RNS Sensing

ROS and RNS are also known to regulate Toll-like receptor (TLR)-mediated signaling (Asehnoune *et al.*, 2004; Ryan *et al.*, 2004; Nicholas and Sumbayev, 2010). Heme is reported to activate a TLR adaptor, MyD88, to induce IL-8 expression in human bronchial epithelial cells (Cosgrove *et al.*, 2011). TLRs play a central role in the initiation of innate immune responses. Until now, 10 human TLRs and 13 mouse TLRs have been discovered. Each TLR recognizes its specific ligand, such as Pam3CSK4 by TLR1/TLR2, double-strand RNA by TLR3, LPS by TLR4, flagellin by TLR5, peptidoglycan by TLR2/6, imiquimod TLR7, R848 by TLR7/TLR8 and bacterial CpG DNA by TLR9. Upon binding of their ligands, the TLRs signal through adaptors and mediators, such as MyD88, TRIF, and IRAK to activate transcription factors, such as NF-κB and AP-1, which result in the release of cytokines and chemokines and the expression of iNOS and antimicrobial peptides (Park *et al.*, 2004). Activation of TLR1, TLR2, and TLR4 induces translocation of TRAF6 leading to ubiquitination of evolutionarily conserved signaling intermediate in Toll (ECSIT), in the macrophage. However, when macrophages are depleted of TRAF6 and ESCIT, the generation of mitochondrial ROS is impaired, thus reducing the bactericidal (West *et al.*, 2011). IL-8 production induced by the TLR4 ligand, LPS, is reduced by treatment with antioxidants, implying that the cellular redox state exerts a potent effect on TLR-dependent cytokine release (Ryan *et al.*, 2004). Bone marrow-derived dendritic cells isolated from iNOS-

deficient mouse respond to TLR2, TLR4, and TLR9 stimulation more strongly than wild-type mouse cells (Giordano *et al.*, 2011).

PAMPs are well known to transduce signals for the expression of immune-responsive genes through TLRs. Host cell-derived DAMPs are also recognized by TLRs. Thus, while TLR-mediated signaling plays a pivotal role in the frontline of innate immune defense against pathogen invasion, uncontrolled TLR signaling may also cause autoimmune diseases (Akira and Takeda, 2004; Mills, 2011).

Hb and TLR Ligand Interactions

Recently, the interrelationship between ROS and the innate immune system in acute lung injury (Xiang and Fan, 2010), CGD (Hartl *et al.*, 2008), hemorrhagic shock, and ischemia (Gill *et al.*, 2010) has been reported. Furthermore, the interactive role of ROS in the cellular response against TLR ligands, such as LPS and flagellin has been elucidated (Qin *et al.*, 2005; Ivison *et al.*, 2010). The direct interaction between Hb and TLR ligands or PAMPs appears to be widespread in occurrence (Yang *et al.*, 2002; Cox *et al.*, 2007; Lin *et al.*, 2012). Since Hb readily forms complexes with PAMPs and DAMPs, it is important to understand the roles of the Hb-PAMP and Hb-DAMP complexes in an innate immune response.

By surface plasmon resonance analysis, we found that both the α and β subunits of Hb possess LPS-binding sites with K_D in the nM range (Bahl *et al.*, 2011). Computational analysis has further shown that interactions between Hb and LPS or Hb and LTA result in a conformational change in the Hb molecule, causing it to convert to the oxidized form, metHb. Consequently, the relaxation of the structural rigidity between Hbα1-β2 and Hbα2-β1 interfaces increases the latent POX activity of Hb (Du *et al.*, 2010). Other studies have shown that coincubation of Hb with multiple TLR ligands or PAMPs synergistically induces cytokines, such as IL-1β, IL-6, IL-8, and TNFα in macrophages (Bodet *et al.*, 2007; Lin *et al.*, 2010). LTA, a TLR2 ligand, induces IL-6 secretion and this is significantly enhanced in the presence of Hb. On the other hand, the TLR4-deficient macrophage shows a lack of production of IL-6 when exposed to the TLR2 ligand. Because LTA is known to bind exclusively to TLR2, the abolished response of TLR4-deficient cells to LTA may be due to a possible link between TLR2 and TLR4 in the cell's response to the LTA-Hb complex (Cox *et al.*, 2007). These results suggest that Hb binds certain PAMPs with specificity to function in nonself recognition and plausibly, to protect the host by producing ROS (Jiang *et al.*, 2007).

Perspectives

Severe hemorrhagic shock and hepatic ischemia-reperfusion injury, which accompany oxidative stress, induce TLR4 expression in the lung and liver (Powers *et al.*, 2006). However, tissue injury and inflammation caused by Hb-mediated oxidative stress was found to be ameliorated by knocking out TLR4 (Chen *et al.*, 2009). In hypoxia inducible factor-1-deficient mice, the TLR2 and TLR6 induction by hypoxia is abolished (Gill *et al.*, 2010). Furthermore, TLR4 is upregulated by the oxidized low-density lipoprotein (LDL) in the macrophage in atherosclerotic plaques (Xu *et al.*, 2001). It was found that colocalization of oxidized LDL with TLR2 and TLR4 in human coronary artery endothelial cells corresponds

to BMP-2 expression, which is an osteogenic factor expressed in calcified human atherosclerotic plaques (Su *et al.*, 2011). All of the above-mentioned observations suggest a close relationship between TLR signaling and oxidative stress. Conceivably, such oxidative stress-related dysfunctions can be triggered by cell-free Hb, which is redox reactive.

Severe oxidative stress-induced cellular apoptosis and necrosis are known to release intracellular DAMPs, such as the heat shock protein, high-mobility group box1 (HMGB1), S100A8, and serum amyloid A, which alert the host innate immune system (Carta *et al.*, 2009). Some DAMPs are known to bind to and activate TLR2 and TLR4 signaling pathways under oxidative stress conditions, for example, the Hb-DAMP complex, such as Hb-HMGB1, was reported to synergistically increase cytokine secretion (Lin *et al.*, 2012). TLR9 also recognizes the non-DNA ligand, namely, hemozoin, which is a hydrophobic heme polymer generated by a malaria parasite, *P. falciparum*. Hemozoin increases the expression of CD40 and CD86 as well as the production of TNFα, IL-12p40, MCP-1, and IL-6 in murine dendritic cells (Coban *et al.*, 2005). In addition to TLR9, it has also been reported that TLR2 and TLR4 respond to the *P. falciparum* glycosylphosphatidylinositols, which induces proinflammatory responses (Coban *et al.*, 2005; Krishnegowda *et al.*, 2005).

The Haptoglobin-Hb (Hp-Hb) complex in the plasma is cleared by CD163, an Hb scavenger receptor on monocytes, whose expression is altered by specific TLR activation (Weaver *et al.*, 2006; Lin *et al.*, 2010). Our recent study also revealed that Apolipoprotein A-1, a major component of high-density lipoprotein, associates with Hb, and the complex is internalized by the recognition of the cell surface scavenger receptor class B type 1 in macrophages and hepatocytes, resulting in the clearance of Hb from the plasma (Du *et al.*, 2012). Although LPS is well known as a TLR4 ligand, it also binds to RP105/MD1 (a class B scavenger receptor), CD36, and DC-SIGN, which are expressed on the lymphocytes, neutrophils, and dendritic cells (Yazawa *et al.*, 2003; Zhang *et al.*, 2006; Baranova *et al.*, 2012). Thus, the human system is well endowed with many nonredundant Hb- or Hb-PAMP- specific binding proteins and cell surface receptors, all of which simultaneously recognize, endocytose, and clear the redox-reactive plasma Hb. Indeed, as a highly potent producer of harmful ROS, it is crucial for blood cells to harbor highly efficient pathways of sensing, suppressing, and rapidly detoxifying the cell-free Hb.

Taken together, all the findings reviewed here corroborate a high likelihood that Hb and other blood components bind to certain PAMPs and DAMPs, and the resulting Hb-PAMP and Hb-DAMP complexes modulate the functions of ligands on the pattern recognition receptors, such as the TLRs, to stimulate different types of immune cells and distinct signaling pathways (Fig. 1). However, current findings on the role of Hb in immune responses have been obtained from studies limited to an *in vitro* cell culture system. These findings restrict a full insight on the biological implications *in vivo*. A thorough systemic investigation is mandatory to better understand the role of Hb in pathogen infection and autoimmune diseases and to provide insights into inflammation mechanisms with a view to the future development of anti-inflammatory therapeutics.

FIG. 1. A model depicting the involvement of extracellular hemoglobin in host cell innate immune responses via Toll-like receptors (TLRs) and other membrane-bound receptors. Extracellular hemoglobin (Hb) released by hemolysis is normally endocytosed by Hb scavengers, such as haptoglobin, scavenger receptor class B type 1 (SR-B1), and CD163 for intracellular degradation. However, excessive levels of extracellular hemoglobin produced under abnormal conditions, overwhelms the Hb scavengers and membrane receptors. The cell-free Hb binds to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). The Hb-PAMP and the Hb-DAMP complexes are recognized by pattern recognition receptors, such as TLRs in various immune cells, triggering proinflammatory responses.
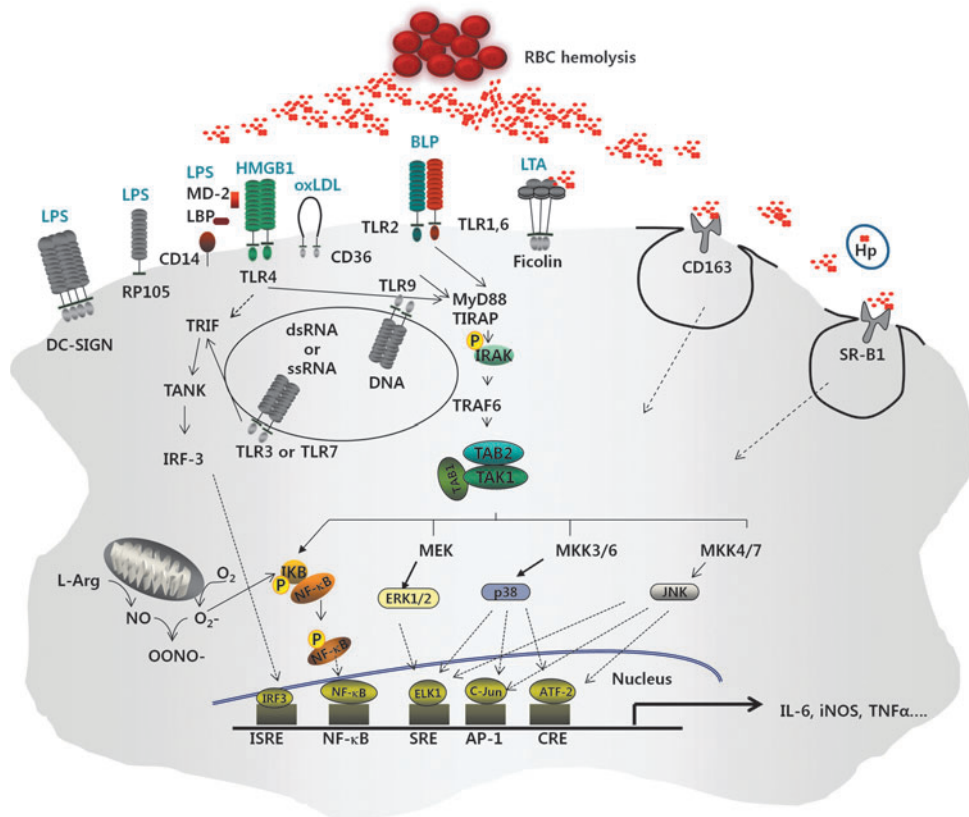
Disclosure Statement

No competing financial interests exist.

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