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Original Article

Response of Neutrophils to Extracellular Haemoglobin and LTA in Human Blood System

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ABSTRACT

Background: Haemolytic infection lyses red blood cells, releasing haemoglobin (Hb) into the plasma. Although recent studies showed that immune cells recognize redox-active cytotoxic extracellular Hb (metHb) bound to pathogen-associated molecular patterns (PAMPs), currently available information is limited to experiments performed in defined conditions using single cell lines. Therefore, a systemic approach targeting primary whole blood cells is required to better understand the cellular immune defence against metHb and PAMPs, when under a haemolytic infection.

Methods: We investigated how human white blood cells, including neutrophils, respond to metHb and lipoteichoic acid (LTA) by measuring reactive oxygen species (ROS), signalling mediators (ERK and p38), NF-κB, cytokines, elastase secretion and cell activation markers.

Findings: metHb activates NF-κB in TLR2-expressing HEK293 cells but not in normal or TLR9-expressing HEK293 cells. Treatment of isolated neutrophils with metHb increased production of ROS and expressions of IL-8, TNFα, and CD11b, which were further enhanced by metHb + LTA complex. While LTA stimulated the survival of neutrophils, it caused apoptotic cell death when complexed with metHb. The activation of neutrophils by metHb + LTA was subdued by the presence of other types of white blood cells.

Interpretation: metHb and metHb + LTA complex are ligands of TLR2, inducing an unconventional TLR signalling pathway. Neutrophils are a highly sensitive cell type to metHb + LTA complex. During a haemolytic infection, white blood cells in the vicinity crosstalk to modulate neutrophil TLR-signalling induced by metHb and LTA.

1. Introduction

Extracellular haemoglobin (Hb) is readily oxidized into metHb, which is highly redox-active and cytotoxic due to its pseudoperoxidase activity (metHb-POX) (Jiang et al., 2007). Dangerous levels of cell-free metHb may persist in the plasma, for example in sickle cell anaemia (1.6 mg/ml) and paroxysmal nocturnal hemoglobinuria (5–20 mg/ml) (Schaer et al., 2013; Hartmann et al., 1966). The plasma metHb as well as pathogen-associated molecular patterns (PAMPs) are released into the plasma in an infection by haemolytic microbes, which may cause systemic inflammatory responses leading to multiple organ dysfunctions. metHb is normally rapidly scavenged by haptoglobin, scavenger receptor class (SR)-B1, and CD163. The internalized metHb undergoes detoxification and degradation (Subramanian et al., 2013; Schaer and Alayash, 2010). However, in severe haemolysis, massive levels of metHb overwhelm the capacity of metHb scavengers leading to excessive production of reactive oxygen species (ROS) by metHb-POX, which perturbs immune homeostasis (Olsson et al., 2012). metHb may bind to other damage-associated molecular patterns (DAMPs) and PAMPs, which are recognized by pattern recognition receptors such as TLRs in various immune cells, to trigger pro-inflammatory responses. When present in the plasma, metHb is a highly redox-reactive major DAMP that threatens the integrity of the white blood cells (WBCs), but its potentials to signal through TLRs are hitherto unclear (Lee and Ding, 2013). The methicillin-resistant strain of *Staphylococcus aureus* is a notorious haemolytic Gram-positive bacterium, which has become a major public health problem (Iwamoto et al., 2013; Stryjewski and Corey, 2014). Lipoteichoic acid (LTA) is the key immunostimulatory component of *S. aureus* that triggers TLR2-activating innate immune system of the host. Hb has been known to form a complex with *S. aureus* LTA to potentiate the immune stimulatory effect of LTA (Hasty et al., 2006). We previously reported the mechanism of ROS production by metHb-POX, showing that binding of LTA to metHb enhances the production of ROS, which not only kills the invading microbe, but is also harmful to the host blood cells (Jiang et al., 2007; Bahl et al., 2011; Du et al., 2010).

Neutrophils are one of the first responding immune cells to an infection. The migration of neutrophils into the site of infection–inflammation is mediated by PAMPs from microbes or DAMPs derived from disrupted host cells. At the site of infection, neutrophils rapidly combat pathogens by unleashing ROS and proteases. Furthermore,

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antimicrobial proteins stored in their cytoplasmic granules are released (Nathan, 2006). The azurophil granules contain myeloperoxidase, defensins, cathepsin G, and elastase, which are released upon activation and degranulation of the neutrophils. Amongst these, elastase which is a major serine protease is involved in various inflammatory responses (Pham, 2006). Neutrophils are known to interact with other leukocytes through cell–cell contact, and they secrete cytokines and chemokines. They modulate dendritic cell maturation and trafficking and are able to cross-present antigens to memory $CD4^+$ T cells as well as to naïve $CD8^+$ T cells, which subsequently amplify $CD8^+$ T cell response to the antigen (Beauvillain et al., 2007). Direct interaction between neutrophils and T cells has been shown in the regression of cancer as well as infectious diseases (Stoppacciaro et al., 1993; Ma et al., 2009). ROS, produced by activated neutrophils, inhibits the effector functions of NK cells, while cytokines such as GM-CSF and IFN-γ released from activated NK cells, prolong the survival of neutrophils in an in vitro system (Costantini and Cassatella, 2011). Moreover, depletion of neutrophils impairs the recruitment of monocytes and lymphocytes to the inflammatory site. On the other hand, the immune suppressive capacity of neutrophils in T cell proliferation during acute systemic inflammation has also been reported (Pillay et al., 2012).

Considering the diverse functions of neutrophils in inflammation, we envisaged that neutrophils would play a significant role in response to plasma metHb during a haemolytic condition. Therefore, we investigated the response of neutrophils and the other types of blood cells, to metHb and LTA. Here, we show that metHb is an endogenous DAMP ligand for TLR2, and that neutrophils are one of the most sensitive cell types responding to (metHb + LTA)-induced production of ROS. Interestingly, this effect is diminished by the presence of other leukocytes, indicating that the white blood cells communicate with each other to modulate cellular response during a haemolytic infection.

2. Materials and Methods

All procedures followed the guidelines of the National University of Singapore Institutional Review Board (NUS-IRB Ref. code: B-14-063E). Buffy coats were obtained from the Blood Bank with appropriate informed consents from the volunteers.

2.1. Cells and Reagents

HEK293 clones expressing human TLR2, TLR9 and *S. aureus* LTA (LTA-SA) were purchased from InvivoGen (San Diego, California, USA). Human Hb, blasticidin, and hygromycin were from Sigma-Aldrich (St. Louis, MO, USA). Buffy coat from healthy donors was obtained from the Blood Bank, National University Hospital, Singapore. Primary blood cells were incubated in 5% CO_2 at 37 °C in HEPES-buffered RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% FBS. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Adult human Hb, which contains 96.5–98.5% HbA1 ($\alpha_2\beta_2$ dimer) and 1.5–3.5% HbA2 ($\alpha_2\delta_2$ dimer), has been verified to be in the metHb state by spectrophotometric scanning between 500 and 700 nm (Du et al., 2010). As the endotoxin concentration in Hb was determined to be 2.86 EU/mg/ml by PyroGene Recombinant Factor C kit (Lonza Inc.), 1 mg/ml of Hb was pre-treated with 5 μg/ml of polymyxin B, which can scavenge 10 EU/ml of endotoxin. The metHb + LTA complex was pre-formed by co-incubating 1 mg/ml metHb with 10 μg/ml LTA for 30 min.

2.2. Isolation of Human Neutrophils and Cellular Assays

Buffy coats obtained from healthy donors were used for the isolation and enrichment of neutrophils with HetaSept™ (Stem Cell Technologies Inc.) according to the manufacturer's instructions. Immediately after isolation, the cells were incubated with different concentrations of metHb, LTA or metHb + LTA for 1 h (or as described in the figure legends), in RPMI medium followed by washing with PBS. For TLR2 blocking assay, 3.8×10^5 neutrophils/well in 12-well plate were preincubated with TLR2 blocking antibody (20 μg/ml, eBioscience) for 1 h and then treated with metHb + LTA complex prepared as described above. For the assessment of intracellular ROS production, the cells were incubated with 1 μM CM-H_2DCFDA for 30 min at 37 °C. After washing with PBS, the fluorescence signal of CM-H_2DCFDA in the cells was measured by flow cytometry (CyAn ADP flow cytometer, Dako).

2.3. Isolation of Human Leukocytes and Stimulation

Human total leukocytes (referred as white blood cells, WBCs) were isolated from buffy coat using HetaSep™ followed by EasySep™ Human Positive Glycophorin A Depletion Cocktail (Stem Cell Technologies Inc.), according to the manufacturer's instructions. Human PBMCs were isolated from buffy coat by Ficoll-Paque density gradient centrifugation. The cells were incubated with metHb, LTA or metHb + LTA (as described above for neutrophils). Activated cells were assessed by flow cytometry for increased expression of activation markers, such as CD86, CD69, CD11b, LFA-1, DNAM-1, and Icam-1. ROS production induced in PBMCs or WBC treated with different stimulators was determined using CM-H_2DCFDA as described above.

2.4. Chemiluminescence Assay for Haemoglobin Pseudoperoxidase (metHb-POX) Activity

The effect of PAMPs on the metHb-POX activity was assessed by measuring $O_2^{\bullet -}$, using a chemiluminescence assay. $O_2^{\bullet -}$-triggered chemiluminescence of Cypridina luciferin analog (CLA) was measured with a luminometer (Promega, Glomax 20/20), and expressed as relative luminescence units per second (RLU/s). metHb incubated with 25 μg/100 μl of each PAMP was added to a substrate mixture containing 20 μM CLA and 5 mM H_2O_2 in 100 μl PBS (pH 7.4) and the resulting chemiluminescence was immediately recorded in real-time up to 150 s, according to Jiang et al. (2007).

2.5. NF-κB Reporter Assay

Stably transfected HEK293/hTLR2-HA, HEK293/hTLR9, and HEK293/vector control cells were plated at the density of 2×10^5/well in 24-well plates and transiently transfected for 24 h with NF-κB luc plasmid

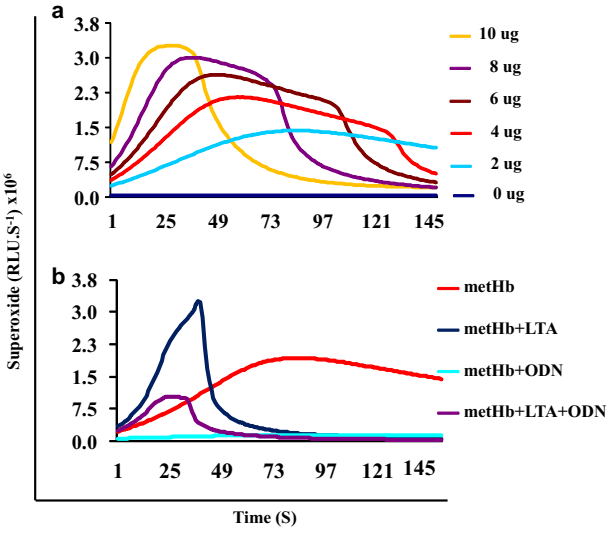

Fig. 1. metHb-POX activity in the presence of LTA. (a) Kinetics of $O_2^{\bullet -}$ production induced by different amounts of metHb. (b) Increase or inhibition of POX activity of metHb (4 μg/ml) by LTA and ODN2395.