# CD163-MEDIATED INNATE IMMUNE

# RESPONSE(S) TO CELL-FREE HEMOGLOBIN

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# DECLARATION

I hereby declare that this thesis is my original work and has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Karthik Subramanian

15 August 2013

# ACKNOWLEDGEMENTS

# TABLE OF CONTENTS

# SUMMARY

Hemoglobin (Hb) is a redox-active molecule capable of generating toxic reactive oxygen species owing to its intrinsic pseudoperoxidase (POX) activity. Inside the red blood cells (RBCs), antioxidants exist to protect Hb from oxidation. However hemolysis, induced during infection, tissue injury/trauma and genetic disorders, releases Hb into the plasma. The uncontrolled oxidative reactions of plasma Hb disrupt the redox balance and impair the immune-responsive blood cells. Therefore, it is crucial to understand how the immune system defends against the cytotoxic Hb. This thesis investigates: (i) the role of scavenger receptors and plasma proteins in conferring protection against cell-free Hb and (ii) pathophysiological role of Hb during hemolytic infections.

Haptoglobin (Hp) is a prominently studied Hb-binding plasma protein, which clears Hb via the monocyte/macrophage scavenger receptor, CD163. However, Hb in Hb:Hp complex still remains POX active. Moreover, Hp is rapidly saturated during severe hemolysis. Hence we explored alternative Hp-independent Hb-detoxification mechanisms. We identified a highly efficient CD163 mediated two-pass Hb detoxification mechanism independent of Hp. Firstly, CD163 directly suppressed the Hb-POX activity *in situ* on the monocyte membrane, and consequently rescued monocytes from Hb-induced apoptosis. Simultaneously, the membrane bound CD163 is also shedded as a soluble protein, sCD163 into the plasma. sCD163 further bound the residual cell-free Hb and the sCD163:Hb complex then interacts with plasma IgG, which bridges the sCD163:Hb complex to FcγR on the monocytes. Subsequently, the sCD163 from the endocytosed sCD163:Hb:IgG complex

undergoes palmitoylation and is recycled via endosomes to the membrane to restore the homeostasis of CD163 in an autocrine cycle. The endocytosed Hb is catabolized by heme-oxygenase-1. Secondly, the sCD163 elicits a paracrine cycle, transactivating the vascular endothelial cells to detoxify Hb.

The elucidation of the detoxification mechanism involving endocytosis of Hb into the monocytes to overcome oxidative damage, prompted us to examine the pathophysiological consequence of Hb-priming on the host cell viability and intracellular bacterial clearance during a hemolytic infection. Under these circumstances, CD163$^+$ macrophages that uptake Hb from the plasma are invaded by opportunistic pathogens. Heme-iron is a nutrient for the invading pathogens, while the Hb-POX activity generates microbicidal ROS, making Hb a double-edged sword. We found that the Hb-primed CD163$^+$ macrophages harbored higher intracellular bacterial load compared to unprimed control cells. Further investigation revealed that the intracellular bacteria elicit a subtle activation of the Hb-POX to evade microbicidal ROS and modulated the MAPK-Bax signaling pathway to downregulate apoptosis, and upregulate anti-inflammatory cytokine production in host cells. *In vivo,* this mechanism may contribute to persistence of infections by using Hb-scavenging phagocytes as mobile vehicles for dissemination and escape from immune surveillance.

The findings of this thesis open new research avenues for the development of sCD163 as a biomarker in hemolytic patients. Further, our identification of Hb-primed CD163$^+$ macrophages as a survival niche for intracellular pathogens provides insights into the interrelationship between hemolysis and infections.

# LIST OF TABLES

## LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic  acid] |
| APAF-1 | Apoptotic-protease-activating factor 1 |
| | |
| Bac | Bacteria |
| Bax | Bcl2 associated X-protein |
| Bcl2 | B-cell CLL/lymphoma 2 |
| BSA | Bovine serum albumin |
| | |
| CCCP | Carbonyl cyanide m-cholorophenylhydrazone |
| CHX | Cycloheximide |
| CLA | Cypridina luciferin analog |
| cFLIP | Cellular caspase-8-like inhibitory protein |
| CFU | Colony forming unit |
| CO | Carbon monoxide |
| $CO_2$ | Carbon dioxide |
| | |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DAMP | Damage-associated molecular pattern |
| Dex | Dexamethasone |
| DMEM | Dulbecco's modified eagle medium |
| | |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal regulated kinase |
| EU | Endotoxin Units |

FACS          Fluorescence activated cell sorting

FADD          Fas-associated death domain

FBS           Fetal bovine serum

FcγR          Fc-gamma receptor

FITC          Fluorescein isothiocyanate


GAPDH         Glyceraldehyde 3-phosphate dehydrogenase

GPI           Glycosylphosphatidylinositol

GSH           Glutathione


h             Hours

HA            Hydroxylamine

HABA          4-hydroxyazobenzene-2-carboxylic acid

Hb            Hemoglobin

HEK293T       Human embryonic kidney 293 cells containing
              SV40 T antigen

HMC           Hemocyanin

HMVEC         Human dermal microvascular endothelial cells

HO-1          Heme Oxygenase-1

Hp            Haptoglobin

Hpx           Hemopexin

HRP           Horseradish peroxidase

HSA           Human serum albumin


IFN           Interferon

IgG           Immunoglobulin G

IL            Interleukin

Isd           Iron-regulated surface determinant

| | |
|---|---|
| $K_{on}$ | Association rate constant |
| $K_{off}$ | Dissociation rate constant |
| $K_D$ | Equilibrium dissociation constant |
| kDa | Kilo dalton |
| | |
| LB | Luria-Bertani |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| | |
| MAPK | Mitogen-activated protein kinase |
| mCD163 | Membrane-associated CD163 |
| MetHb | Methemoglobin |
| MFI | Mean fluorescent intensity |
| mg | Milligram |
| min | Minutes |
| ml | Millilitre |
| MOI | Multiplicity of infection |
| MyD88 | Myeloid differentiation primary response protein-88 |
| | |
| NAC | N-acetyl cysteine |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced) |
| NLR | Nod-like receptor |
| NO | Nitric oxide |
| NRAMP-1 | Natural resistance associated macrophage protein-1 |
| | |
| OD | Optical density |
| oxLDL | Oxidized low-density lipoprotein |

| | |
|---|---|
| *P. aeruginosa* | *Pseudomonas aeruginosa* |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate-buffered saline |
| PFA | Paraformaldehyde |
| PI | Propidium iodide |
| PLA | Proximity ligation assay |
| PMA | Phorbol 12- myristate 13-acetate |
| PMB | Polymyxin B |
| PMCA | Plasma membrane calcium ATPase |
| PNH | Paroxysmal nocturnal hemoglobinuria |
| POX | Pseudoperoxidase |
| PRR | Pathogen recognition receptors |
| | |
| RBCs | Red blood cells |
| rFC | Recombinant Factor C |
| RIG-1 | Retinoic acid-inducible gene-1 |
| RLR | RIG-like receptor |
| RLU | Relative luminescence units |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RPMI | Rosewell Park Memorial Institute |
| RU | Response Unit |
| | |
| s | Seconds |
| *S. aureus* | *Staphylococcus aureus* |
| *S. typhimurium* | *Salmonella typhimurium* |
| sCD163 | Soluble CD163 |
| SDS | Sodium dodecyl sulfate |
| siRNA | Small interfering RNA |

| | |
|---|---|
| SOD | Superoxide dismutase |
| SPR | Surface plasmon resonance |
| SRCR | Scavenger receptor cysteine rich |
| Sup | Supernatant |
| | |
| TBS | Tris-buffered saline |
| TIR | Toll/interleukin-1 receptor |
| TIRAP | TIR-domain containing adaptor protein |
| TLR | Toll-like receptor |
| TMRE | Tetramethyl rhodamine ethyl ester |
| TNF-$\alpha$ | Tumor necrosis factor-alpha |
| TRAM | TRIF-related adaptor molecule |
| TRIF | TLR-receptor associated activator of interferon |
| v/v | volume/volume |
| w/v | weight/volume |

## LIST OF PRIMERS

| Name | Sequence (5' to 3') | Purpose |
|---|---|---|
| CD163-FW | CCC AAG CTT GCC ACC ATG AGC AAA CTC AGA ATG GTG CTA C | Forward primer for CD163 with HindIII restriction site |
| CD163-RW | CCG CTC GAG TTA AAT TCC CAT TTT CCT TTT CAG TG | Reverse primer for CD163 with XhoI restriction site |
| CD163: 478-1bp | TCA TTT CCA AAT TGG ATC CAT CTG A | Sequencing primer to verify CD163 clone |
| CD163: 770-1540bp | TTG CTC AAA GGG AGC AGA TCT GAG CC | Sequencing primer to verify CD163 clone |
| CD163: 1541-2310bp | TTC TAT GCA GGG AAT TAC AGT GTG GCA | Sequencing primer to verify CD163 clone |
| CD163: 2310-3033bp | TCT GCT CAT TTT GGG AAA GGA ACA GGG C | Sequencing primer to verify CD163 clone |
| CD163: 3033bp | CGC TGG GGC CAT AGT GAG TGT GGG CA | Sequencing primer to verify CD163 clone |

# CHAPTER 1

# INTRODUCTION

## 1.1 Hemolysis and cell-free hemoglobin (Hb)

### 1.1.1 Hb is a pro-oxidant

Hemoglobin (Hb) is one of the most critical and well-studied proteins in human physiology owing to its function in respiration as the oxygen transporter. Under normal physiological conditions, Hb is compartmentalized within the reducing environment of the red blood cells (RBCs) by efficient antioxidants (Buehler & D'Agnillo, 2010). Therefore, in healthy individuals, the extracellular concentration of Hb is minimal and tightly controlled. However, under pathological conditions such as during infection (Berkowitz, 1991), trauma (Sadrzadeh et al, 1987), hematological disorders (Olsson et al, 2012) and blood transfusion (Berseus et al, 2013), high concentrations of extracellular Hb accumulate in the plasma (Muller-Eberhard et al, 1968; Schaer et al, 2013).

Extracellular Hb is toxic to several tissues including vascular (Balla et al, 1991b; Balla et al, 1993), myocardial (Burhop et al, 2004), renal (Nath et al, 2000; Rabiner et al, 1967) and the central nervous system (Regan & Panter, 1993; Sadrzadeh et al, 1987). The source of Hb mediated toxicity, *in vivo* and *in vitro,* originates at the heme prosthetic group, which is hydrophobic and hence readily permeates the cell membrane (Tracz et al, 2007). The iron centre of the heme group is reactive and can interact with numerous ligands leading to the formation of ferric (Fe(III)) and ferryl (Fe(IV)) radical species (Alayash,

1

2004). As a result, the oxidative nature of free Hb leads to the production of ROS and induces cellular and tissue damage by oxidation of lipids, nucleic acids and amino acids (Everse & Hsia, 1997).

**1.1.2 Pseudoperoxidase activity of Hb**

Hb is a tetramer consisting of two $\alpha$ and two $\beta$ polypeptide chains (**Figure 1.1**). Each polypeptide chain has an iron-containing heme prosthetic group inside the hydrophobic pocket and carries one oxygen atom per heme group. The heme iron normally exists in the reduced ferrous (Hb-Fe(II)) state but can spontaneously undergo autoxidation, leading to the formation of ferric state metHb (Hb-Fe(III)) as well as reactive oxygen species (ROS) like superoxide ion ($O_2^-$). Inside the RBCs, autoxidation of Hb is kept to a minimum by the efficient reductase system involving catalase and superoxide dismutase, which efficiently neutralize ROS and catalyze the reduction of ferric iron back to the ferrous state. The $O_2^-$ undergoes dismutation reaction to produce hydrogen peroxide ($H_2O_2$). In the presence of the endogenous and/or exogenous hydrogen peroxide, a catalytic cycle between the ferric (Hb-Fe(III)) and ferryl (Hb-Fe(IV)) heme is initiated (**Figure 1.2**), in which hydrogen peroxide is eliminated in a peroxidase-like manner (Alayash, 1999). This is known as the pseudoperoxidase (POX) cycle of Hb. The ferryl Hb formed is unstable and can release free heme and heme degradation products (Nagababu & Rifkind, 2000). The ROS released during the reactions of metHb with hydrogen peroxide causes cellular and tissue damage by oxidation of lipids, proteins and nucleic acids (Everse & Hsia, 1997).
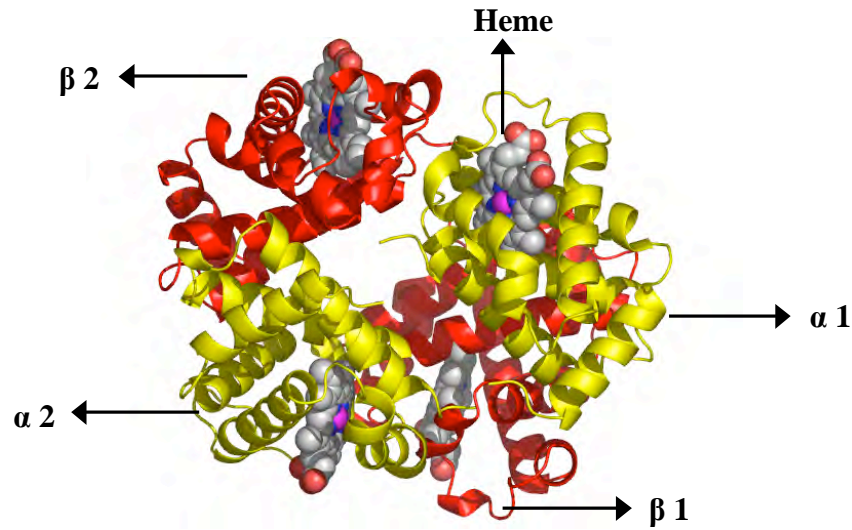
**Figure 1.1: Tetrameric structure of human hemoglobin.** The alpha chains (α1, α2) are shown in yellow while the beta chains (β1, β2) are in red. The four heme groups are shown as spheres. Adapted with modifications from the protein data bank, PDB ID code- 1HGA.
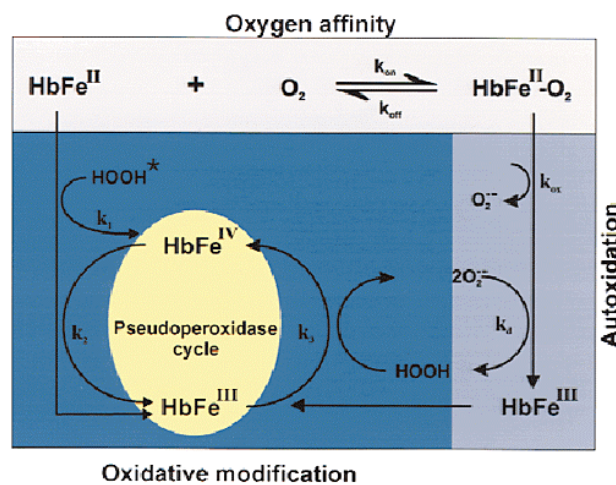


**Figure 1.2: Pseudoperoxidase activity of Hb.** The catalytic cycling of Hb associated heme iron between the ferrous (Hb-Fe(II)), ferric (Hb-Fe(III)) and ferryl (Hb-Fe(IV)) oxidation states in the pseudoperoxidase cycle (yellow). HOOH: hydrogen peroxide. Figure adapted from (Alayash, 1999) with permission from the Nature publishing group.

**1.1.3 Mechanisms of Hb toxicity**

Several pathophysiological conditions such as sickle cell disease, hemolytic infection, paroxysmal nocturnal hemoglobinuria and trauma are characterized by extensive hemolysis. Under these circumstances, the excessive level of plasma Hb wreaks havoc in the vasculature and triggers an array of toxic effects. The principal mechanisms leading to the adverse clinical effects of extracellular Hb are: 1) extravascular translocation of Hb; (2) scavenging of nitric oxide (NO) and oxidation reactions; (3) release of the iron-containing porphyrin group, hemin and (4) molecular signaling effects of hemin. These mechanisms are summarized in **Figure 1.3**.

Mechanism I: Extravascular translocation of Hb

Free Hb in the plasma released during hemolysis is unstable and exists in a dynamic equilibrium between the tetramer ($\alpha 2 \beta 2$) and the heterodimer ($\alpha \beta$) states with a predominant shift towards the dimer configuration at low concentrations. The relatively smaller molecular size of the $\alpha \beta$ dimer (32 kDa) allows it to penetrate the endothelial barrier and cause damage to the tissues beneath.

Mechanism II: NO scavenging and oxidation reactions

In the vasculature, NO is produced in the endothelial cells lining the blood vessels by a constitutively expressed synthase enzyme system. NO regulates the vascular homeostasis and tone, leukocyte adhesion and platelet aggregation along with a host of other biochemical functions (Moncada, 1992).

4

The interaction of Hb with NO occurs mainly via two reactions:

(1) NO dioxygenation of oxy-Hb

$$Hb-(Fe(II))-O_2 + NO \longrightarrow (Hb-(Fe(III)OONO^.) \longrightarrow Hb-(Fe(III)) + NO_3^-$$
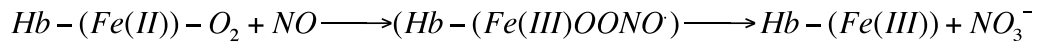
(2) Iron nitrosylation of NO deoxy-Hb

$$Hb-Fe(II) + NO \longrightarrow Hb(NO)$$

Therefore, as a result of interaction with NO, the plasma Hb has been known to induce vasoconstriction and hypertension (Olson et al, 2004). In addition to vasoconstriction, another effect of Hb-NO interaction is the generation of Hb-(Fe(III)) with the tissue parenchyma. Accumulation of the unstable Hb-(Fe(III)) within the tissues could lead to the release and transfer of hemin to other proteins/lipids along with toxicity driven by free heme.

Apart from NO scavenging, the pro-oxidant, Hb has been reported to release large amounts of peroxides into the extracellular space during inflammation and ischemia-reperfusion (Reeder, 2010). *In vitro*, the reactions of Hb with peroxide leads to the formation of ferric (Hb-Fe(III)), ferryl (Hb-Fe(IV)) and associated superoxide radicals. The resulting superoxide radicals not only cause local amino acid oxidations within Hb but also transfer radicals to other lipoproteins (Jia et al, 2007; Miller et al, 1997). The net result of these reactions is release of free hemin and globin chain precipitation, which can ultimately lead to tissue damage (Vallelian et al, 2008).

Mechanism III: Release of free hemin

The oxidative reactions of Hb leads to the formation of (Hb-Fe(III)), which is unstable and releases free hemin. The hydrophobic nature of free hemin allows for the transfer of its reactive porphyrin to cell membrane and plasma proteins and lipids. One of the most abundant toxic end products of hemin is the oxidized low-density lipoprotein (oxLDL) (Balla et al, 1991a). Oxidation of LDL and associated inflammatory effects of hemin represents the ability of Hb to induce vascular injury (Jeney et al, 2002; Nagy et al, 2010).

Mechanism IV: Molecular signaling effects of hemin

The hydrophobic hemin released by Hb can readily permeate the cell membrane and bind to several receptors, transcription factors and enzymes and alter gene expression and cellular activation. One of the most well defined interaction is the binding of hemin to the transcription factor, Bach-1, which regulates the expression of heme-oxygenase-1 (HO-1) and other anti-oxidative enzymes in response to high intracellular levels of hemin (Ogawa et al, 2001). In addition, hemin has also been reported to activate Toll-like receptor (TLR) signaling especially TLR-4 in certain animal models (Lin et al, 2012a) and bind to the nuclear hormone receptor, REV-ERB, which regulates circadian rhythm, metabolism and adipogenesis (Raghuram et al, 2007). Some studies have also documented inhibition of the proteasome by intracellular hemin although predominantly using *in vitro* assays (Santoro et al, 2012; Tanaka & Ichihara, 1989).

**Figure 1.3: Principal mechanisms of Hb toxicity.** Schematic representation of the pathways by which free Hb released during hemolysis induces toxicity. Figure adapted from (Schaer et al, 2013) with modifications.

**1.2 Hb is a DAMP and triggers innate immune response**

During severe hemolysis, free Hb released from the ruptured RBCs can reach excessively high concentrations in the plasma (e.g. 0.6 mg/ml in sickle cell anaemia (Schaer et al, 2013); 0.27-0.45 mg/ml in malaria (Sharma et al, 2012) and 0.5-2 mg/ml in paroxysmal nocturnal hemoglobinuria (Rother et al, 2005)) and becomes a redox-active damage-associated molecular pattern (DAMP). The ROS generated by Hb perturbs the immune cells to maintain homeostasis. In this section, we will discuss the role of Hb in the innate immune system, focusing on the possible interactions among Hb, pathogens and immune cells.

**1.2.1 Overview of the innate immune system**

**1.2.1.1 Pathogen recognition receptors recognize microbes and trigger immune signaling**

The immune system continually co-evolves with the pathogens to develop a variety of defense mechanisms to counter the microbial invaders. The immune system in vertebrates consists of two components- the innate, and the adaptive immune response. The innate immune system is a rapid and non-clonal defense consisting of germline-encoded receptors, while the adaptive immunity is more pathogen specific and based on antigen-specific receptors in the clonally selected B- and T-lymphocytes (Medzhitov & Janeway, 1997). Innate immune recognition is mediated by structurally diverse set of receptors called pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include bacterial cell wall components (peptidoglycan, lipopolysaccharides (LPS), lipotechoic

acid (LTA)), nucleic acids (DNA, RNA) and flagellin. PRRs can be present as circulating plasma proteins (e.g. complement proteins, ficolins, C-reactive proteins), as receptors on the cell membrane (e.g. Toll like receptors (TLRs), scavenger receptors) or in the intracellular compartments (Nod-like receptors (NLRs) and some TLRs) (Medzhitov & Janeway, 1997);(Zhang et al, 2010). **Figure 1.4** represents some examples of PRRs and the PAMPs they recognize.

The innate immune recognition of microbes by PRRs relies on three key principles. Firstly, PAMPs are chemical signatures unique to the microbes and are not present in the host and hence prevent response to self-antigens. Secondly, PRRs recognize chemical signatures that are conserved amongst a variety of microbes enabling a single receptor to detect variety of pathogens (e.g. receptor for LPS can recognize any gram-negative bacterium). Third, the PAMPs are essential for the survival of the microbes and are hence retained throughout the evolution of the microbe.

In mammals, TLRs are the best-characterized examples of PRRs (Beutler, 2004). TLRs are either localized at the plasma membrane and recognize bacterial and viral surface components or recognize viral and bacterial nucleic acids exposed within the endosomal compartments. For example, membrane localized TLR-4 and TLR-2 recognize bacterial LPS and lipopeptide respectively. On the other hand, endosomal localized TLR- 7 and TLR-9 recognize viral single-stranded RNA and CpG DNA motifs respectively. Upon binding to the respective ligand, TLRs trigger intracellular signaling pathways leading to the production of inflammatory cytokines such as TNF-$\alpha$, IL-6, IL-12, type-I-interferon (IFN) and chemokines (Figure 1.4). TLRs contain a cytoplasmic signaling domain called TIR (Toll/interleukin-1

receptor) which recruits adaptor molecules such as myeloid differentiation primary response protein 88 (MyD88), TIR-domain containing adaptor protein (TIRAP)/Mal, Toll-receptor-associated activator of interferon (TRIF) and TRIF-related adaptor molecule (TRAM) leading to the nuclear translocation of transcription factors such as NF-κB.
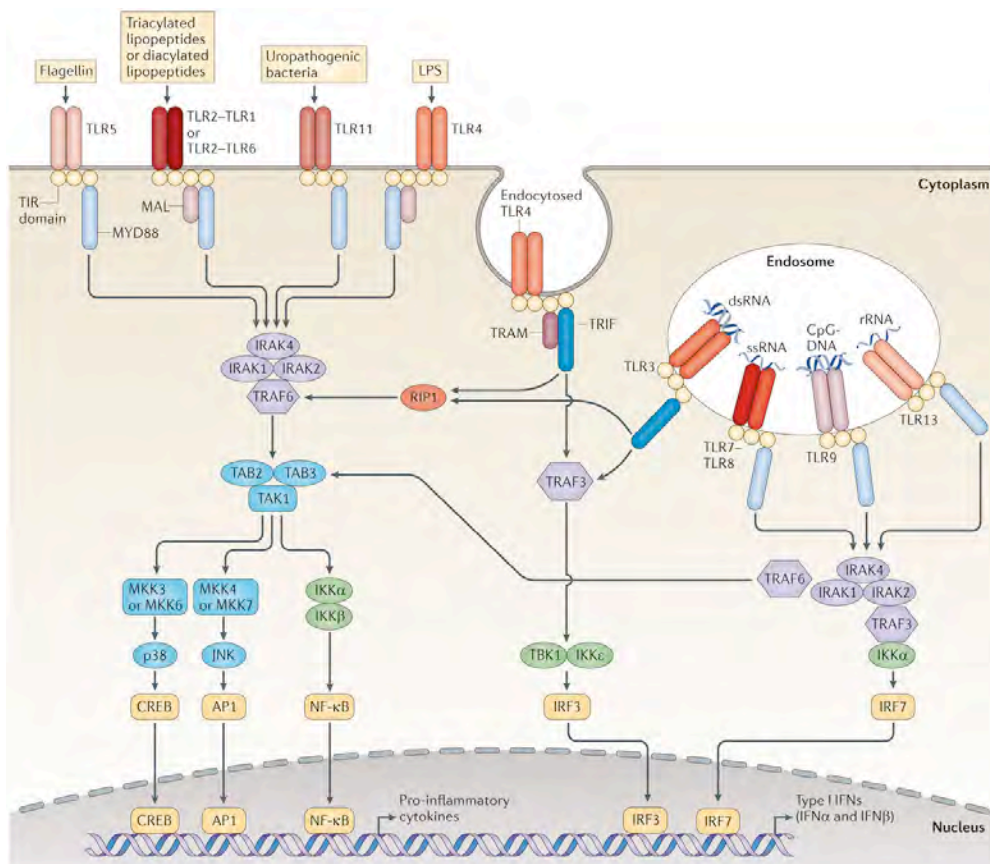


**Figure 1.4: TLR-signaling and activation of inflammatory response.** Upon recognition of the respective ligand, TLRs initiate signaling via adaptor proteins to activate inflammatory cytokine response. Figure adapted from (O'Neill et al, 2013) with permission from the Nature publishing group.

The cytokines and chemokines elicited by TLR signaling recruit effector cells such as neutrophils to the site of infection and activate antigen-presenting cells such as dendritic cells, which then induce the activation of antigen-specific T-lymphocytes. Hence, TLRs link innate and adaptive innate immune response.

Apart from the PRRs expressed on the cell surface, there is a cytosolic detection system for intracellular PAMPs. Among these are retinoic acid-inducible gene-1 (RIG-1) like receptors (RLRs) and nucleotide binding oligomerization domain (NOD) like receptors (NLRs). RLRs specifically detect viral RNA in the cytoplasm and induce anti-viral type-1 IFN production. NLRs such as NOD-1 and NOD-2 recognize bacterial peptidoglycan and induce inflammatory cytokines like IL-1β, IL-18 and IL-33 through the formation of "inflammasome" which involves activation of caspases (Meylan et al, 2006).

### 1.2.1.2 Apoptosis regulates cell death during infection

The pro-inflammatory cytokine production elicited by PRR-PAMP signaling leads to programmed cell death called apoptosis, during which an infected cell commits suicide to prevent the spread of infection to neighboring cells. There are two main pathways involved in apoptosis- an extrinsic pathway initiated by cell-surface receptors, and an intrinsic pathway that is initiated by the mitochondria in response to intracellular stress **(Figure 1.5)** (Hotchkiss & Nicholson, 2006). Cellular proteases, caspase-8 and caspase-9 mediate the extrinsic and the intrinsic apoptotic pathways respectively(Roy & Nicholson, 2000). Both caspase-8 and caspase-9 finally converge at caspase-3, which is

the crucial effector caspase involved in the final common pathway of apoptosis.

Ligands of the death receptors, like TNF-$\alpha$ and CD95L activate caspase-8, while intracellular stress such as ROS, chemotherapeutic agents and damaged DNA activate caspase-3. Upon engagement of the death receptors such as CD95 (FAS) by their ligands, signaling via the adaptor protein, FAS-associated via death domain (FADD) triggers the cleavage of the inactive, procaspase-8 into active caspase-8. Active caspase-8 cleaves BID and the truncated BID (tBID) activates the intrinsic pathway, thereby interconnecting the two pathways. Cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP) prevents the activation of caspase-8.

In the intrinsic pathway, pro-apoptotic factors like BCl-2 associated X protein (BAX) decrease the mitochondrial membrane potential, leading to the release of cytochrome c. Together with apoptotic-protease-activating factor 1 (APAF-1), cytochrome c activate the pro-caspase-9 into functional caspase-9. Second mitochondria-derived activator of caspase (SMAC) is also released from the mitochondria and blocks the inhibition of caspase-9 by the inhibitors of apoptosis (IAPs). Anti-apoptotic factors like B-cell lymphoma (BCL-2) and BCL-X$_L$ inhibit the loss of mitochondrial membrane potential and cytochrome c release. Hence, the fine balance between the pro- and the anti-apoptotic factors regulate cellular apoptosis.
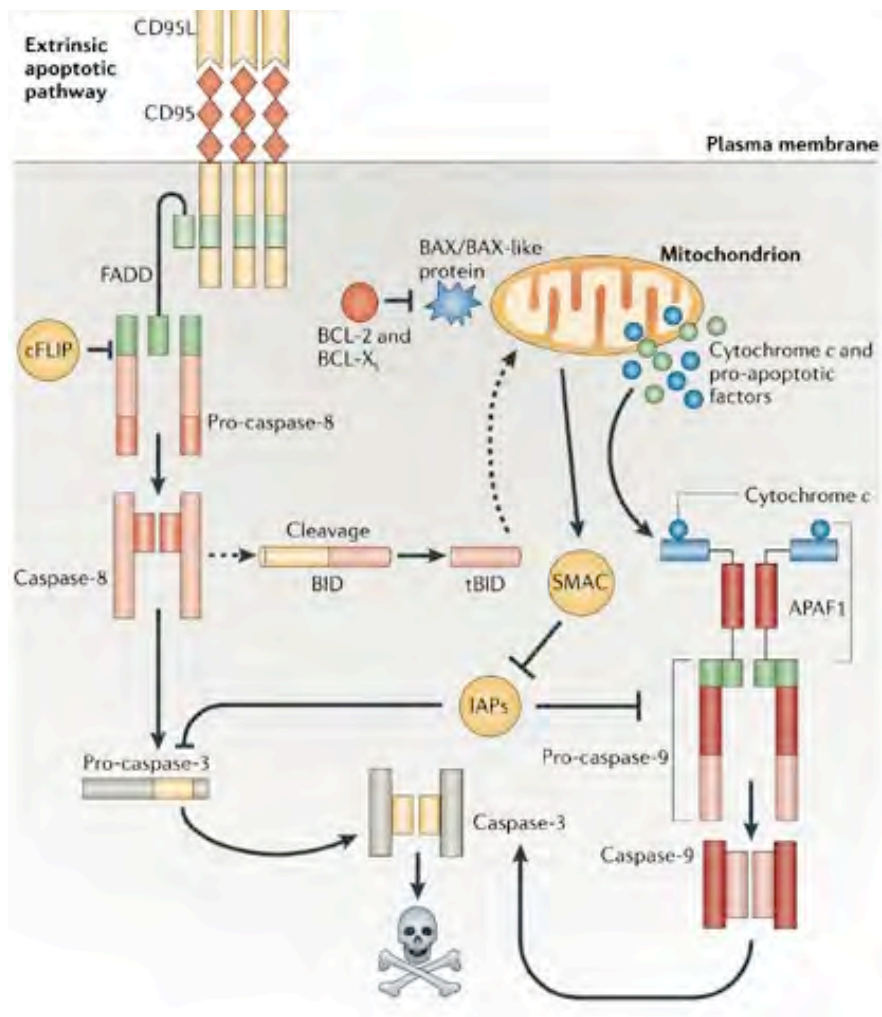
**Figure 1.5: The extrinsic and intrinsic pathways of apoptosis.** Apoptosis occurs via two pathways- the extrinsic pathway, which is initiated by death receptors, and the intrinsic pathway, which is initiated at the mitochondria. Caspase-8 mediates the extrinsic pathway, while caspase-9 mediates the intrinsic pathway. Both caspase-8 and caspase-9 finally converge at a common effector protease, caspase-3. Figure adapted from (Hotchkiss & Nicholson, 2006) with permission from the Nature publishing group.

**1.2.2 Hb is a DAMP and evokes immune response**

Apart from PAMPs, certain endogenous host-derived molecules such as Hb also trigger the innate immune system, leading to inflammation (Lee & Ding, 2013). Intraerythrocytic Hb, which normally transports oxygen, becomes a DAMP when released into the extracellular environment during hemolysis. The redox-active Hb generates cytotoxic ROS, which perturbs the immune cells in the blood. ROS are known to trigger TLR-mediated signaling (Gill et al, 2010) and are implicated in several diseases such as acute lung injury, chronic granulomatous disease (Hartl et al, 2008; Xiang et al, 2010). Heme, the prosthetic group of Hb has been shown to activate TLR-4 and mediate inflammation by signaling via the MyD88-TRIF pathway (Lin et al, 2012a). Further, Hb synergistically activates inflammation with other PAMPs such as LPS and LTA (Bodet et al, 2007; Cox et al, 2007). In the next section, the interactions between Hb and PAMPs are further discussed in detail.

**1.2.3 Interrelationship between Hb, a DAMP and microbial PAMPs – implications on innate immunity**

During a hemolytic infection, Hb liberated into the plasma is in contact with the intruding pathogen and/or it's associated PAMPs. (Kaca et al, 1994) reported that Hb could form a stable complex with LPS and consequently activated the redox activity of Hb leading to the production of metHb. Recent study in our lab has found that both $\alpha$ and the $\beta$ subunits of Hb possess high affinity LPS-binding sites (Bahl et al, 2011). Hb has also been found to augment the host-immune response to LPS (a conserved Gram-negative bacterial PAMP) during infections. Free Hb enhanced the production of TNF-

α induced by LPS in human mononuclear cells (Carrillo et al, 2002) and tissue factor production by endothelial cells (Roth, 1994). (Kaca & Roth, 1995) found that Hb contaminated with LPS augmented the activation of the complement cascade. Furthermore, co-administration of Hb amplifies the mortality induced by LPS in animal models (Goff et al, 1999). A potential complication associated with the Hb:LPS interaction is the effect of LPS contamination of Hb in blood transfusion products (Su et al, 1997), which presents a barrier to the use of cell-free Hb in blood substitutes. Apart from LPS, Hb has been shown to recognize other TLR ligands (Lin et al, 2010) and other DAMPs (Lin et al, 2012b) and generate microbicidal ROS. The induction of IL-6 production by LTA, a TLR-2 ligand is significantly increased upon incubation with Hb (Cox et al, 2007).

## 1.2.4 Cells involved in the clearance of plasma Hb

Monocytes and macrophages are the key cells that are involved in the clearance of extracellular Hb within the hemorrhagic plagues (Schaer & Buehler, 2013). These are the primary cells in the blood that express the Hb-scavenger receptor, CD163 and have been implicated in the clearance and detoxification of plasma Hb (Kristiansen et al, 2001). In the next section, the structure and function of CD163 are discussed in further detail.

## 1.2.5 Hb and free heme scavenging mechanisms

A network of plasma proteins, enzymes and scavenger receptors exists to promptly clear free Hb from the plasma during mild to moderate hemolysis **(Figure 1.6)**. The principal clearance mechanism involves transport to the

liver parenchyma whereupon heme oxygenase 1 (HO-1) in the macrophages breaks down the porphyrin ring into bilirubin, carbon monoxide and iron.

The most studied Hb scavenging protein in the plasma is haptoglobin (Hp), which binds to free Hb with high affinity (Levy et al, 2010). Binding of Hp to Hb sequesters the redox-active Hb in the vasculature and prevents translocation across the endothelial barrier and into the kidney. Hp has also been shown to alter the redox reactions of Hb by stabilizing the higher oxidation states of Hb (Hb-Fe(IV)) and preventing the radical transfer to other molecules (Banerjee et al, 2012; Cooper et al, 2013). As a result of this protection, oxidation of the globin moiety of Hb is reduced thus preventing its degradation. Owing to the protective effect of Hp against cell-free Hb, Hp has been clinically used to treat patients with burn injuries, trauma and hemolytic anaemia (Schaer et al, 2013).

Hemopexin (Hpx) is a hemin-binding plasma protein that forms the second line of defense against free hemin released from metHb. Although several plasma proteins such as albumin and lipoproteins bind to hemin, Hpx is the most effective ($K_d < 10^{-13}$ M) in sequestering hemin in an oxidatively inert conformation (Gutteridge & Smith, 1988; Tolosano et al, 2010). In the plasma, Hpx binds to free hemin released from metHb and prevents the oxidation of lipoproteins and interaction of hemin with cell-surface receptors such as TLR-4 (Figueiredo et al, 2007). Endocytic receptors expressed on the surface of macrophages and monocytes clear the Hb:Hp and heme:Hpx complexes from circulation. In particular, the scavenger receptor, CD163 has been shown to recognize Hb:Hp complex from the plasma (Kristiansen et al, 2001; Schaer et al, 2007). The heme:Hpx complex is cleared by the CD91

receptor on hepatocytes (Hvidberg et al, 2005). The above protective mechanisms protect against the toxic effects of circulating cell-free Hb.
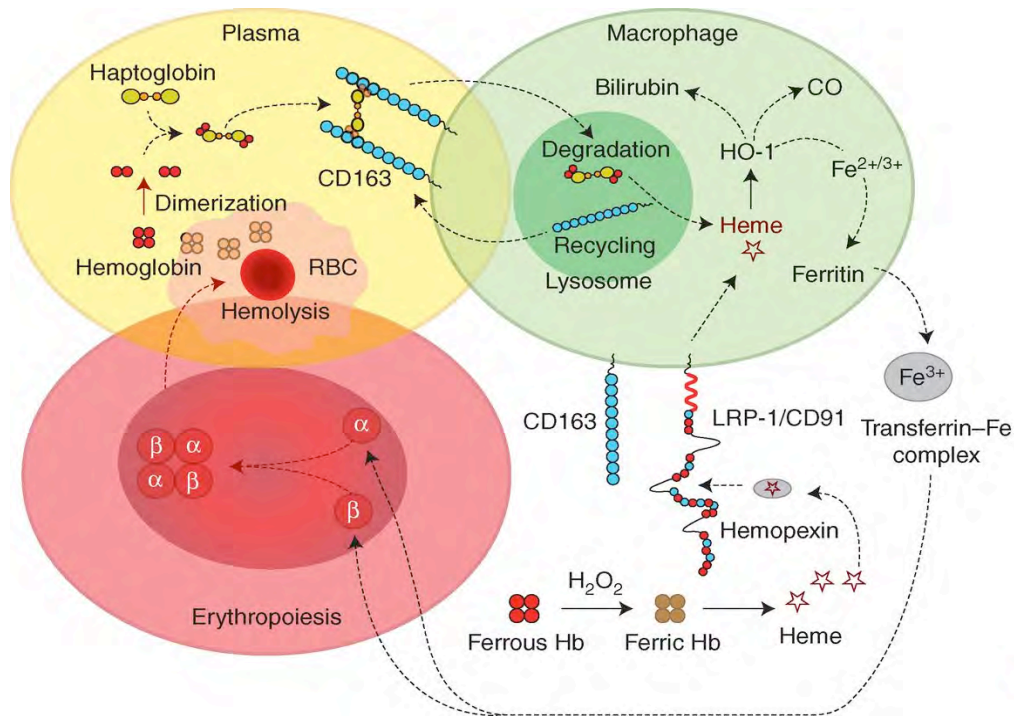


**Figure 1.6: Hb and free heme scavenging mechanisms.** Free Hb released into the plasma during hemolysis and tissue injury. In the extracellular compartment, free Hb reacts with peroxides ($H_2O_2$) and undergoes oxidation into ferric Hb, which is unstable and releases free heme. In the plasma, Hb is sequestered by Hp and the Hb:Hp complex is internalized by scavenger receptor, CD163. Within the macrophage, Hb is catabolized by heme-oxygenase-1 (HO-1) into bilirubin and carbon monoxide (CO). Scavenging buffer systems such as hemopexin (Hpx) binds and detoxifies free heme and the heme:Hpx complex is recognized by the LDL-receptor related protein (LRP-1)/CD91. Figure adapted from (Schaer & Buehler, 2013).

**1.2.5.1 CD163 as a monocyte/ macrophage scavenger receptor**

Structure and function

CD163 was initially isolated, cloned and sequenced from the splenic macrophages of a hairy cell leukemia patient (Law et al, 1993). CD163 is a type I transmembrane protein (~130 kDa) and belongs to the scavenger receptor cysteine rich (SRCR) superfamily of proteins. It comprises of an extracellular domain of ~1003 amino acids, containing 9 SRCR repeats, a hydrophobic transmembrane domain of ~24 amino acids and a short cytoplasmic domain of ~49 amino acids (**Figure 1.7**). Depending on the length of the cytoplasmic tail, four splice variants were identified in the human monocyte cDNA library (Law et al, 1993).



**Figure 1.7: Schematic representation of CD163 structure.** CD163 contains nine scavenger receptor cysteine rich (SRCR) domains towards the N-terminal while the C-terminus is intracellular and connected by a short transmembrane domain.

The two major functions of CD163 reported so far in the literature are: a) receptor for Hb:Hp complex and b) regulate the innate immune response during infection (Graversen et al, 2002). Membrane bound-CD163 has been shown to bind Hb:Hp complex and Hb in the plasma via the third SRCR domain in a calcium-dependent manner (Madsen et al, 2004; Schaer et al, 2006). Upon binding to CD163, the Hb:Hp complex is internalized into the macrophages, wherein HO-1 degrades the heme into bilirubin, CO and iron

and induces anti-inflammatory cytokines such as IL-10 (Akila et al, 2012). Apart from binding to Hb:Hp complex, CD163 has also been shown to bind both Gram-negative and Gram-positive bacteria and induce cytokine production (Fabriek et al, 2009). However, the exact nature of the bacterial ligands and the intracellular signaling pathways remains to be understood.

CD163 has been implicated in the anti-inflammatory immune response. Firstly, macrophages expressing CD163 are predominant during the resolution phase of inflammation and are found in wound-healing tissues (Zwadlo et al, 1987). Secondly, CD163 expression is strongly upregulated by anti-inflammatory mediators such as glucocorticoid and IL-10 (Schaer et al, 2001) in a macrophage subtype called alternatively activated macrophages. The anti-inflammatory IL-10 produced by CD163 expressing macrophages has been shown to inhibit T-lymphocyte proliferation (Akila et al, 2012). Moreover, the catabolic end products of heme like bilirubin and CO have been shown to exert anti-oxidative and anti-inflammatory effects (Otterbein et al, 2000).

Expression and regulation

Expression of CD163 is restricted to cells on the monocyte/macrophage lineage (Akila et al, 2012). In particular, CD163 is expressed on peripheral blood monocytes and more mature tissue macrophages such as Kupffer cells in the liver, red-pulp macrophages in the spleen and cortical macrophages of the thymus (Van den Heuvel et al, 1999). CD163-positive macrophages are predominantly found in healing wound tissues post-inflammation (Philippidis et al, 2004).

The expression of CD163 is regulated by several factors such as glucocorticoids and cytokines. The promoter region of CD163 has several potential glucocorticoid receptor binding sites, which could explain the strong induction of CD163 upon glucocorticoid stimulation of monocytes and macrophages. Pro-inflammatory stimuli such as cytokines (interferon-$\gamma$, tumor necrosis factor-$\alpha$) and LPS have been shown to downregulate CD163 mRNA and protein (Ritter et al, 1999). In contrast, anti-inflammatory stimulus like IL-10 has been shown to upregulate the expression of CD163. Hence, CD163 is subjected to regulation by both pro- and anti-inflammatory stimuli, suggesting a crucial role of CD163 in regulating immune responses.

<u>Proteolytic shedding of CD163</u>

In addition to the membrane-bound form of CD163 (mCD163), a soluble form of CD163 exists in the plasma and tissue fluids (Moller et al, 2002). Proteolytic cleavage of CD163 by metalloproteinases releases the extracellular portion of CD163 containing the 9 SRCR domains and has a molecular mass identical to membrane-CD163. To distinguish the two forms of CD163, this thesis refers to the soluble form of CD163 released upon proteolytic cleavage as sCD163 and the full-length native membrane bound form as mCD163. Inflammatory stimuli such as LPS and oxidative stress have been shown to induce shedding of CD163 from monocytes (Hintz et al, 2002; Timmermann & Hogger, 2005). The concentration of soluble CD163 (sCD163) is upregulated in patients with sepsis, myeloid leukemia and Gaucher disease (Moller et al, 2004).

Clinical Relevance of CD163

CD163-expressing cells have been associated with many diseases. The restricted expression of CD163 on monocytes and macrophages makes it an ideal target for the delivery of therapeutics specifically to these cells. CD163-expressing macrophages in the gut have been identified in patients with the inflammatory disease, spondyloarthropathy (Baeten et al, 2002). CD163-specific antibodies have been used to stain synovial macrophages in rheumatoid arthritis patients (Fonseca et al, 2002). CD163 also serves as a marker for the macrophages in atherosclerotic lesions (Ratcliffe et al, 2001) and has been proposed to play a role in lesion formation (Schaer, 2002). In addition, infection by viruses such as human immunodeficiency virus type-1 (HIV-1) and porcine reproductive and respiratory syndrome virus (PRRSV) has been associated with the CD163-high anti-inflammatory macrophages (Chihara et al, 2012; Karniychuk et al, 2013). Patients with sepsis and other inflammatory diseases have elevated levels of sCD163 in the plasma (Matsushita et al, 2002; Moller et al, 2002). Hence, both the membrane-bound and soluble forms of CD163 play important roles in infection and inflammation.

## 1.3 Significance of hemolysis and Hb during pathological conditions

Hemolysis and elevated concentrations of extracellular Hb in the plasma are associated with various diseases and genetic abnormalities such as hemolytic infections (e.g. malaria, hemorrhagic fevers), hemolytic anaemia (e.g. paroxysmal nocturnal hemoglobinuria, autoimmune hemolytic anaemia), and tissue injury (e.g. intraventricular brain hemorrhage, chronic leg ulcers). In

this section, we will focus on the role of cell-free Hb and heme during hemolytic infections.

### 1.3.1 Hb-iron is a nutrient source for pathogens

Iron is an important micronutrient for all forms of life. Hb, being the most abundant iron source in humans, is an attractive source of iron for the intruding pathogens (Pishchany & Skaar, 2012). In view of the importance of iron in host-pathogen interactions, both the host and the pathogen have co-evolved mechanisms to sequester iron. Apart from being a source of the nutrient iron, pathogens such as *Plasmodium* (malarial parasite), utilize Hb as a source of amino acids (Francis et al, 1997). Hemoglobin is sequentially digested by a series of proteases expressed within the digestive vacuole of the parasite (Skinner-Adams et al, 2010).

Iron acquisition by pathogens

Hemolytic bacteria, such as *Staphylococcus aureus* are known to express hemolysin that ruptures the RBCs and releases free Hb (Vandenesch et al, 2012). Released Hb and its heme group are captured by receptors that are either expressed on the surface of the bacteria or secreted (Hammer & Skaar, 2011). Siderophores are high affinity iron-binding molecules secreted by the bacteria to acquire iron from the iron-sequestering proteins of the host such as transferrin and lactoferrin. *S. aureus* produces two distinct siderophores, staphyloferrin A and staphyloferrin B (**Figure 1.8**). The genes for siderophores are controlled by the iron-dependent ferric uptake regulator (Fur), and are highly expressed in iron-limiting environments (Friedman et al,

2006). Apart from siderophores, *S. aureus* possesses the iron-regulated surface determinant (Isd) system, which mediates the acquisition and transport of heme across the bacterial cell wall and plasma membrane. The family of Isd receptors includes IsdA, IsdB, IsdC, IsdD, IsdE, IsdG, and IsdI, which are able to bind to Hb, Hp:Hb complexes, and heme (Haley & Skaar, 2012). IsdA, IsdB, IsdH and IsdC contain the conserved 'near iron transporter' (NEAT) domains, which mediates heme and hemoprotein binding (Haley & Skaar, 2012). The IsdB receptor, which functions as the Hb-receptor, has been shown to have greater affinity to human Hb compared to other species (Hammer & Skaar, 2011). After being transported across the membrane through the Isd receptors, heme eventually reaches the cytoplasm where it is degraded to release iron, which is eventually incorporated into the bacterial heme containing proteins (Hammer & Skaar, 2011). Apart from bacteria, several pathogens such as *Leishmania* and *Trypanosoma* have also evolved mechanisms to acquire iron from Hb (Carvalho et al, 2009; Vanhollebeke et al, 2008).
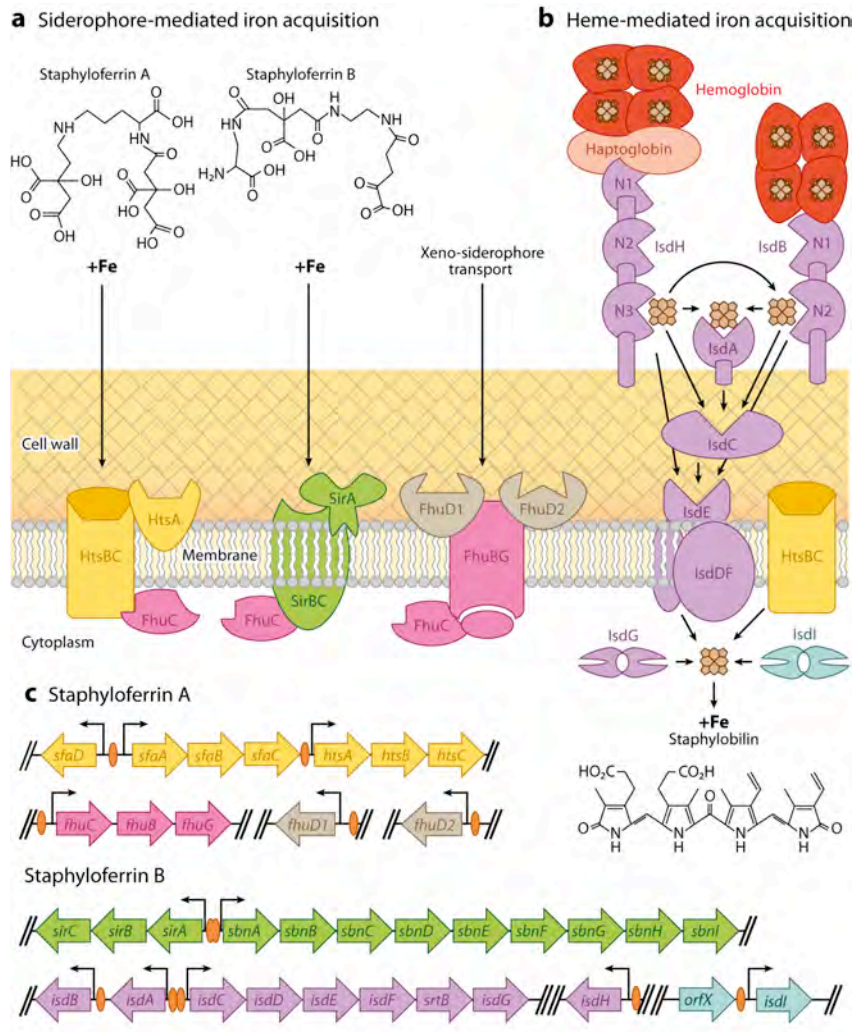
**Figure 1.8: Mechanisms for iron acquisition by *Staphylococcus aureus.* (a**) *S. aureus* produces two siderophores, staphyloferrin A and staphyloferrin B that bind to extracellular iron with high affinity. HtsA lipoprotein and HtsBC permease import staphyloferrin A, while the SirA lipoprotein and SirBC permease import staphyloferrin B. (**b**) Heme acquisition by *S. aureus* through the Isd system. IsdH binds to Hb:Hp complexes, while IsdB binds Hb directly. Heme is passed through IsdH, IsdB, IsdA, IsdC, IsdE and finally across the membrane through either the IsdDF or the HtsBC permeases. Bacterial heme-oxygenases, IsdG and IsdI then degrade the intracellular heme to release iron. (**c**) The promoter regions of the staphyloferrin genes contain consensus binding regions for Fur (orange) which regulates the expression of these genes under iron-limiting conditions. Figure adapted from (Hammer & Skaar, 2011).

24

Iron sequestration by the host

Free Hb and heme released during hemolysis have dual roles in innate immunity. Firstly, the heme is a source of nutrient iron for pathogens. Secondly, the redox-active Hb generates cytotoxic ROS. Hence, the host has evolved proteins to sequester Hb and heme/iron from the intruding pathogens, as well as to protect from oxidative damage. In the plasma, iron is bound by the transport protein, transferrin and transported into the macrophages, where in the iron is either stored intracellularly by ferritin (Parrow et al, 2013) or incorporated into iron-containing proteins. Lactoferrin, found in the mucosal surfaces and plasma, binds to iron with higher affinity than transferrin and retains it under acidic conditions (Aisen & Leibman, 1972). Other plasma proteins such as Hp and hemopexin (Hpx) bind to plasma Hb and heme respectively(Parrow et al, 2013). All these Hb- and iron-chaperone proteins are acute phase proteins whose levels are regulated during infection. Due to the tight regulation of free iron by the chaperone proteins, under normal physiological conditions, free iron in the body fluids is maintained at an extremely low concentration of $10^{-18}$ M, which cannot sustain bacterial growth (Bullen et al, 1978). During infection and inflammation, hepcidin, an iron-regulated hormone is upregulated and further reduces the free iron concentration in the serum. Thus, the host has evolved several iron and Hb-chaperone proteins in order to deprive the intruding pathogens of the nutrient iron.

**1.3.2 Hb senses pathogen and produces antimicrobial ROS**

Hemolytic pathogens secrete cytolytic toxins to release Hb from the RBCs, and subsequently proteolyse the extracellular Hb to release the heme-iron and utilize it as a nutrient source. However, the host employs the redox activity of Hb as an anti-microbial defense against the invading pathogen. This is exemplified by the findings that Hb elicits a broad spectrum of anti-microbial defense (Mak et al, 2007; Parish et al, 2001). In particular, Hb has been shown to recognize and bind to bacterial PAMPs such as LPS, LTA (Du et al, 2010; Jiang et al, 2007). Upon binding to the PAMP, the POX activity of Hb is activated leading to the production of microbicidal ROS in the vicinity of the bacteria (Du et al, 2010). Apart from PAMPs, proteolysis of Hb by microbial proteases such as subtilisin A, type XIV proteases, elastase from bacteria, proteinase K from fungi (Jiang et al, 2007) releases POX-active peptides, which anchor onto the surface of the microbe and release toxic ROS (**Figure 1.9**) (Du et al, 2010). This anti-microbial function of Hb is evolutionarily conserved in the hemocyanin (HMC) of the invertebrate, horseshoe crab implying the central importance of this mechanism in mediating innate immune defense against blood-borne pathogens.
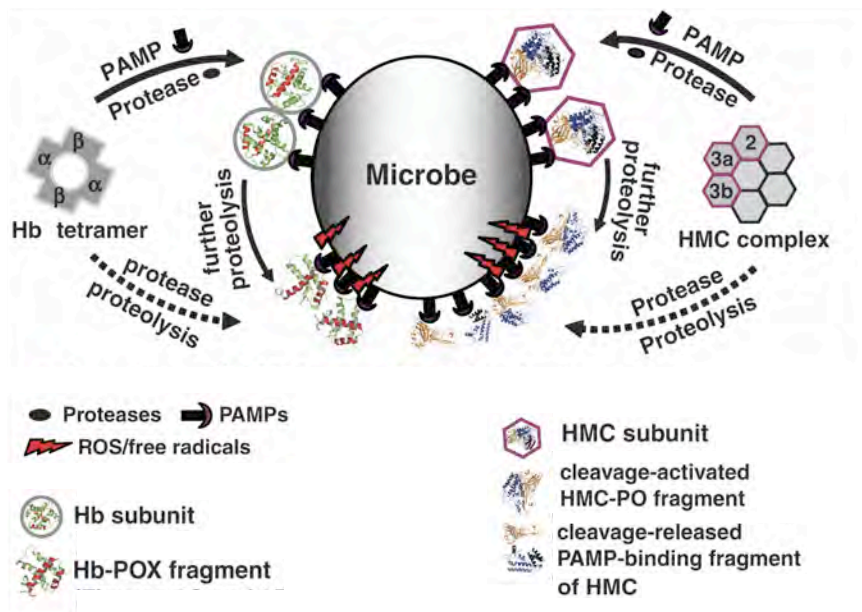
**Figure 1.9: Hb senses pathogen and releases ROS.** Tetrameric or the proteolysed forms of Hb and HMC bind to PAMPs on the microbial surface and generate ROS to kill the pathogen. Figure adapted from (Du et al, 2010) with permission from the Nature publishing group.

The tetrameric form of Hb ($\alpha_2\beta_2$), as well as the individual subunits (Hb-$\alpha$ and Hb-$\beta$) display anti-microbial property. Hemocidins are a class of anionic peptides of Hb released during the proteolytic cleavage of Hb and exhibit anti-bacterial activity (Sheshadri & Abraham, 2012). *In vivo* degradation of Hb exposes the hydrophobic anti-microbial peptides, which act on the negatively charged bacterial membrane (Mak et al, 2000). Examples of hemocidins include the residues 1-23, 33-61, 35-56 of Hb-$\alpha$ and 111-146 of Hb-$\beta$ (Fogaca et al, 1999; Froidevaux et al, 2001; Liepke et al, 2003). Hence, Hb acts like a PRR to sense blood-borne pathogens and instantly generates ROS to subjugate the intruding pathogen.

### 1.3.3 Association between hemolysis and intracellular infections

Hemolysis is induced during both infectious (malaria, *Staphylococcal* infections) as well as non-infectious diseases (sickle cell disease, paroxysmal nocturnal hemoglobinuria, trauma, tissue injury) and is characterized by high levels of the cytotoxic Hb in the plasma. In collaboration with plasma proteins like Hp, scavenger receptors such as CD163, localized on monocytes and macrophages endocytose Hb from the plasma to prevent Hb-induced oxidative damage. Opportunistic pathogens such as *Staphylococcus aureus* are known to usurp Hb to utilize the heme-iron for its survival (Skaar et al, 2004). Hence, the uptake of Hb by macrophages during episodes of hemolysis, presents an opportunity for intracellular pathogens to feed on the intracellular Hb for the nutrient iron. Consistently, several epidemiological studies have reported that patients with hemolytic disorders are predisposed to infections (Berkley et al, 2009; Bronzan et al, 2007; Scott et al, 2011) especially by intracellular bacteria (Mabey et al, 1987).

The mechanism(s) proposed so far, which account for the higher susceptibility of hemolytic patients to intracellular infections involve monocyte and macrophage dysfunctions. For example, hemolysis may impair monocyte and macrophage function by direct adhesion of infected RBCs (Urban & Roberts, 2002), through accumulation of hemozoin with the cells (Schwarzer et al, 1992) or by impairment of IL-12 production (Roux et al, 2010). Another study has shown that resistance to intracellular infections is impaired due to the free heme liberated during hemolysis (Cunnington et al, 2012). However, the missing link that associates hemolysis and intracellular infections remains to be identified.

**1.4 Hypothesis and research objectives**

This thesis explores the innate immune response(s) against cell-free Hb. Severe hemolysis induced during hemolytic infection, tissue injury/trauma and other genetic disorders releases excessive amounts of Hb into the plasma. The intrinsic Hb-POX activity generates cytotoxic ROS, which is damaging if not rapidly detoxified and cleared from the circulation. The interrelation between ROS and the innate immune system in several diseases prompts our systematic analysis of the innate immune defense mechanisms against the DAMP, Hb.

Although plasma Hp has been reported to bind and mediate internalization of Hb via the scavenger receptor, CD163 on the monocytes during moderate hemolysis, the redox activity of the heme-iron is not completely suppressed and the Hb:Hp complex is still redox active (Azarov et al, 2008; Kapralov et al, 2009). Moreover, during severe hemolysis, Hp is rapidly bound and exhausted by the excessively high concentrations of free Hb in the plasma (0.5-2 mg/ml) (Kormoczi et al, 2006). In addition, Hp knockout mice and patients with anhaptoglobinemia do not display complete morbidity to hemolysis, suggesting that under conditions of Hp depletion, alternative parallel mechanisms operate to detoxify and clear the residual Hb. Thus, intriguing questions remain unanswered; for example, a) the functional significance of CD163:Hb interaction on the redox activity of Hb and b) the fate of the shed sCD163 under severe hemolytic conditions. The intricate association between hemolysis and susceptibility to intracellular infection prompts the investigation of the pathophysiological role of internalized Hb towards the survival and growth of iron-dependent intracellular bacteria.

Based on the above observations, we **hypothesize** that firstly in the transient absence of Hp, the host presumably relies on CD163 and other plasma proteins to protect from oxidative damage by Hb-POX and secondly, opportunistic bacteria may utilize the internalized Hb to silently survive inside Hb-loaded macrophages. The major **objectives** of this thesis are thus to: (i) decipher the direct effect of CD163 and plasma proteins on the Hb-POX activity and the consequential cell-survival when Hp is depleted; (ii) investigate the pathophysiological role of internalized Hb towards the survival of intracellular bacteria (**Figure 1.10)**.

Overall, the findings from this thesis will provide important clues to design safer Hb-based blood substitutes and better treatment options for opportunistic bacterial infections in hemolytic patients.
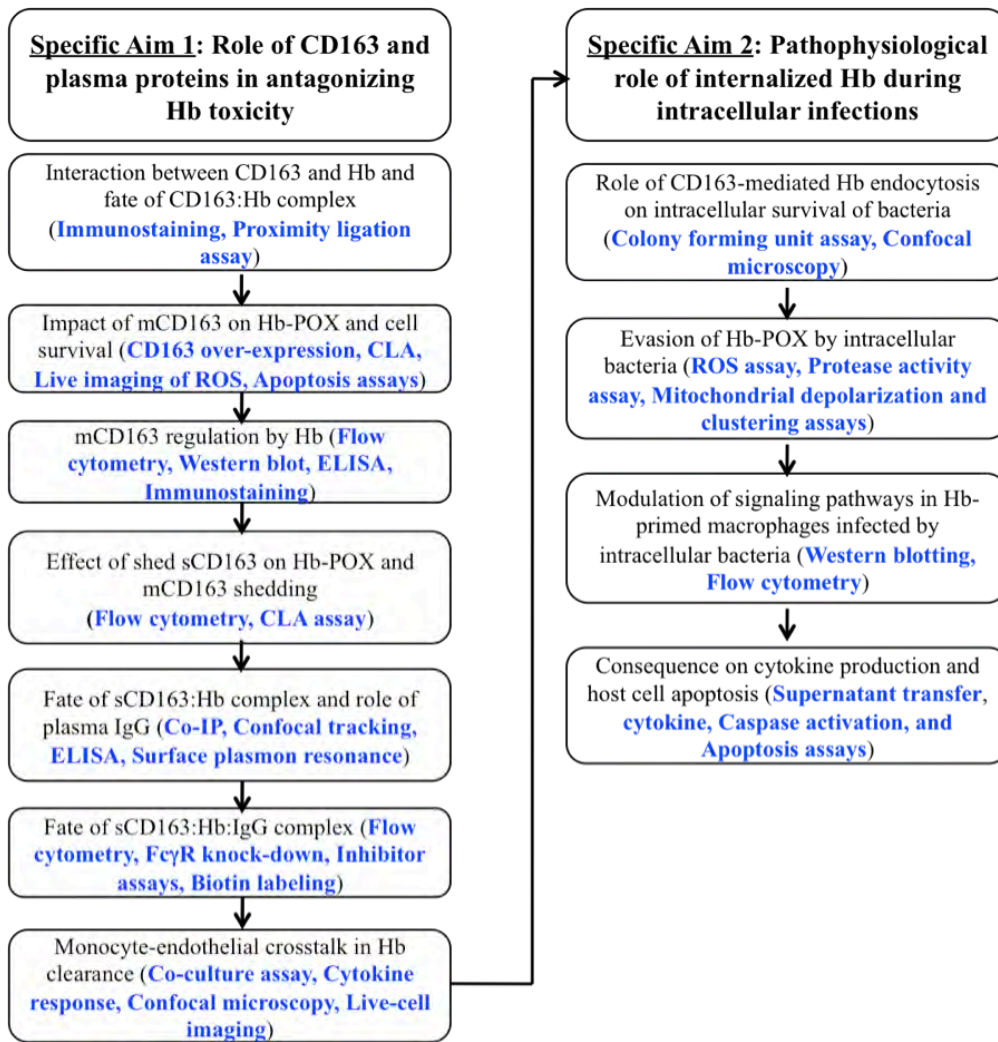
**Figure 1.10: Flowchart showing the specific aims and the experimental strategies used to test the hypothesis.**

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Preparative Methods

### 2.1.1 Reagents

The purified human Hb, subtilisin A, rabbit polyclonal anti-human Hb, rabbit anti-human IgG and the protein synthesis inhibitor (cycloheximide, CHX) were obtained from Sigma. The endocytosis and recycling inhibitors, chlorpromazine and monensin, respectively, were from Calbiochem. Palmitoylation and myristoylation inhibitors, 2-bromopalmitate and 2-hydroxymyristic acid, respectively, were purchased from Sigma. Mouse monoclonal anti-human FcγRI (CD64) and goat polyclonal anti-human CD163 were purchased from R&D Systems. Purified mouse anti-human FcγRIII (CD16) and mouse anti-human FcγRII (CD32) were from BD Pharmingen. Rabbit anti-heme-oxygenase-1 (HO-1), rabbit anti-pERK, rabbit anti-Bax and rabbit anti-p-p38 MAPK were from Cell Signaling Technology. Rabbit anti-NRAMP-1 (Natural resistance-associated macrophage protein) was purchased from Abcam. Mouse monoclonal anti-plasma membrane calcium ATPase (PMCA) was from Thermo Scientific. Mouse monoclonal antibodies against GAPDH and Bcl2 were from Santa Cruz. Goat anti-rabbit, goat anti-mouse and rabbit anti-goat secondary antibodies conjugated to horseradish peroxidase (HRP), were from Dako. Supersignal West Pico chemiluminescent substrate was from Thermo Scientific (Pierce). Pyrogen-free water was from Baxter Healthcare. RPMI-1640 and DMEM were purchased from Gibco,

Invitrogen. EndoGRO-LS complete medium for culturing endothelial cells were purchased from Millipore. Luria Bertani (LB) broth was from Difco, BD Biosciences. High-grade HyClone fetal bovine serum (FBS), with extremely low levels of endotoxin (<0.3-0.6 EU/ml) was purchased from Thermo Scientific. Bovine-serum albumin (BSA) was from Merck. Prolong Gold Antifade Reagent were from Invitrogen. Maxisorp plates for ELISA were purchased from NUNC. All the other chemicals used were of molecular biology grade from Sigma, unless indicated otherwise.

### 2.1.2 Bacterial strains and culture

The laboratory strains, *Staphylococcus aureus* PC1839 (extracellular protease, V8-active; kanamycin-resistant), *Salmonella enterica* serovar Typhimurium LT2 and *Pseudomonas aeruginosa* strain PAO1 (extracellular protease, elastase-active) were used in this study. The cultures were frozen in LB broth containing glycerol (20% v/v) at $-80°C$. *S. typhimurium* and *P. aeruginosa* cultures were maintained on LB agar while Kanamycin (50 μg/ml) supplemented LB was used as the selective media for *S. aureus*.

### 2.1.3 Depyrogenation of glassware and preparation of pyrogen free buffers

To minimize endotoxin contamination, all the glasswares were depyrogenated by baking at $200°C$ for 2 h. All the buffers and media were prepared using pyrogen-free water (Baxter Healthcare) and handled under sterile conditions.

### 2.1.4 Cell cultures and human primary monocytes

Histiocytic lymphoma cells, SU-DHL-1 (obtained from DSMZ, Germany), also described as monocytic M5-type cells, the only human cell line which expresses high levels of CD163 (Law et al, 1993), THP-1 and U937 cells (human leukemic monocyte lymphoma cells) and Jurkat cells (a human T cell lymphoblast cell line) were cultured in HEPES-buffered RPMI-1640 containing 10% FBS at $37^{o}C$ in an atmosphere of 5% $CO_2$. The U937 cells were differentiated into $CD163^+$ macrophages by stimulation with $1x10^{-8}$ M phorbol myristic acid (PMA) and $2.5x10^{-7}$ M dexamethasone (Dex) for 3 days in complete medium. The basis of using dex-treated U937 cells was to upregulate the expression of the hemoglobin (Hb) receptor, CD163 and differentiate them into macrophages which will uptake Hb (Yamazaki et al, 2007).

HEK293T and HepG2 cells were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS. Human dermal microvascular endothelial cells (HMVEC), which expresses FcγRII (CD32) (Groger et al, 1996) were cultured in EndoGRO-LS complete medium on gelatin coated flasks. Cell cultures were maintained at exponential growth rate by replacing media every 2 to 3 days and cells were provided fresh media the day before they were used for experiments.

Primary human monocytes were purified from buffy coat by Ficoll-Paque (GE Healthcare) density gradient centrifugation followed by immuno-magnetic cell sorting using the Human Monocyte Enrichment Kit (StemCell Technologies) according to the manufacturer's instructions. All experiments

involving primary monocytes were performed according to the guidelines on ethics and biosafety (Institutional Review Board, reference code 11-095E). Prior consent was obtained from all the healthy human donors before collection of blood and processing of the buffy coat.

Monocyte-endothelial co-culture

To study the potential cross talk between monocytes and endothelial cells towards the clearance and detoxification of plasma Hb, CD163$^+$ human primary monocytes were co-cultured with HMVEC cells (CD163$^-$, Fc$\gamma$RII$^+$). Briefly, confluent HMVEC cells were washed twice with PBS and incubated with freshly isolated CD163$^+$ primary monocytes or CD163$^-$ THP-1 cells at a ratio of 1:1 in PBS for 45 min with or without Hb, and prepared for immunostaining. For cytokine assays, the cells were co-cultured for 24 h in serum-free RPMI 1640 in the presence or absence of Hb, and the supernatants were collected for ELISA.

## 2.1.5 Preparation of sCD163 and IgG

For purification of sCD163 from cell culture supernatant, 50 µg of anti-CD163 in binding buffer (TBS, pH 7.5) was conjugated to protein A-Sepharose beads by overnight incubation with rotation at 4$^o$C. Unbound antibody was washed twice with binding buffer and the anti-CD163 bound to the beads was cross-linked to Sepharose by incubating for 60 min in a cross-linking buffer (50 mM dimethyl pimelimidate in 200 mM triethanolamine, pH 8.9). The beads were washed twice and incubated with the culture supernatant for 60 min at room

temperature. After three washes, bound sCD163 was eluted using 2.5% acetic acid into tubes containing neutralization buffer (1 M Tris-HCl, pH 12.0).

For purification of IgG from healthy human serum, protein G Sepharose (GE Healthcare Life Sciences) was incubated with 5 µl of serum (containing ~ 10 mg/ml IgG (Stoop et al, 1969)) diluted to 400 µl in binding buffer (20 mM sodium phosphate, pH 7.0). The mixture was rotated on an end-to-end rotator overnight at 4°C. The beads were washed twice with binding buffer, and the bound IgG was eluted using 0.1 M glycine-HCl, pH 2.7, into tubes containing neutralization buffer (1 M Tris-HCl, pH 12.0). All experiments were validated using IgG purified from at least three different healthy donors.

## 2.1.6 Extraction of native cell-membrane proteins

The native membrane proteins from $2 \times 10^6$ SU-DHL-1 cells or primary monocytes were extracted using a native membrane protein extraction kit (ProteoExtract; Calbiochem) according to the manufacturer's instructions. Briefly, cells were washed twice with ice cold PBS and incubated for 10 min on ice under gentle agitation with 2 ml of ice-cold extraction buffer I supplemented with protease inhibitor cocktail. The insoluble material was pelleted by centrifugation at 16000xg for 15 min at 4°C and the supernatant enriched in cytosolic proteins was frozen at -80°C. The cell pellet was then incubated with 1 ml of ice-cold extraction buffer II supplemented with protease inhibitor cocktail for 30 min on ice, with gentle agitation. The insoluble material was pelleted by centrifugation at 16000xg for 15 min at 4°C

and the supernatant enriched in membrane proteins was collected and used immediately or frozen at -80$^{o}$C.

### 2.1.7 FITC conjugation to Hb

Hb was conjugated to fluorescein isothiocyanate (FITC) using the FITC labeling kit (Thermo Scientific) to visualize and quantitate its endocytosis into the monocytes by confocal microscopy and flow cytometry. The labeling procedure was carried out in accordance with the manufacturer's instructions. Briefly, 40 µl of borate buffer (0.67 M) was added to 0.5 ml of 2 mg/ml Hb in PBS. Next, 0.5 ml of Hb in borate buffer was added to the vial containing FITC and mixed thoroughly by pipetting. The labeling reaction mixture was incubated for 60 min at room temperature protected from light. Unbound FITC was removed by adding 250 µl of the labeling mixture onto spin columns containing purification resin, mixed and spun down at 1000xg for 30-45 s to collect the FITC-conjugated Hb. The labeled protein was aliquoted and frozen at -30$^{o}$C.

### 2.1.8 Biotinylation of sCD163

Sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Sulfo-NHS-S-S-biotin; Pierce) was used for biotin labeling of sCD163 to track its subcellular localization and distinguish it from mCD163. Briefly, 2 µg/ml of sCD163 in PBS was incubated with 20-fold molar excess of Sulfo-NHS-SS-Biotin at room temperature for 60 min. Excess biotin reagent was removed

using ultracentrifugal spin columns (with MWCO at 10 kDa Amicon Ultra-0.5) and the biotin-conjugated sCD163 was buffer exchanged to PBS (pH 7.4).

To measure the level of biotin incorporation in sCD163, a mixture of 4-hydroxyazobenzene-2-carboxylic acid (HABA) and avidin was used. Due to its higher affinity for avidin, biotin displaces HABA from its interaction with avidin and the absorbance at 500 nm decreases proportionately. 20 µl of biotinylated sCD163 was added to 180 µl of the HABA/avidin mixture in a 96-well plate and absorbance at 500 nm was measured using a microplate reader (Biotek). The change in the absorbance of the HABA/avidin mixture before and after addition of biotinylated sCD163 ($\Delta A_{500}$) was calculated using the equation below.

$$\Delta A_{500nm} = (A_{500nm} HABA/avidin) - (A_{500nm} HABA/avidin/biotin - sCD163)$$

The concentration of biotin in the reaction mixture was measured as follows:

$$mmol biotin/ml = \Delta A_{500nm} \div (34000 \times 0.5)$$

The level of biotin incorporated into sCD163 was quantified to be 18 biotin molecules per sCD163 molecule using the equation below:

$$Biotin/sCD163 = (mmol biotin/ml) \div (mmol CD163/ml)$$

**2.2 Analytical Methods**

**2.2.1 Superoxide and ROS detection**

**2.2.1.1 Chemiluminescence assay for superoxide detection**

**Principle**

The generation of superoxide ($O_2^-$) by Hb was monitored by the chemiluminescence of Cypridina Luciferin analog (CLA), 2-methyl-6-phenyl-3,7 dihydroimidazo [1,2-a] pyrazin-3(7H)-one. The chemiluminescent dye, CLA emits light upon reacting with superoxide ions (Kawano et al, 2002; Nakano, 1990). A time course profile of the change in the chemiluminescence of CLA indicates that product formation reaches a peak and then declines due to substrate consumption. The relative luminescence units per second (RLU/s) is proportional to the POX enzyme activity and used here as the parameter of analysis of Hb-POX. The CLA dye specifically measures superoxide, enabling the detection of the Hb-POX activity in the presence of hydrogen peroxide.

**Cell-free superoxide production by Hb**

Native Hb or Hb activated by treatment with subtilisin A (refer section 3.1.1.1) was incubated with the SU-DHL-1 membrane extract (enriched in CD163) with or without pretreatment with 0.1 µg/ml anti-CD163. The reaction mixture along with 20 µM CLA and 5 mM $H_2O_2$ in 100 µl PBS was subjected to the chemiluminescence assay. The intensity of the CLA chemiluminescence was measured for 120 s at a 1 s interval using the Glomax 20/20 Luminometer (Promega).

The slope of the curve was used to calculate the POX activity. CD163$^-$ HEK293T cell membrane extract served as negative control.

### *In situ* chemiluminescence assay with cells

CD163$^+$ and CD163$^-$ HEK293T cells were incubated with subtilisin A-activated Hb for up to 10 min, washed twice and then subjected to the CLA assay. The reaction mixture has $2 \times 10^5$ cells, 20 µM CLA and 5 mM $H_2O_2$ in 100 µl PBS.

### 2.2.1.2 Intracellular ROS detection using CMH$_2$DCFDA dye

The ROS generated within the monocytes was measured using the cell permeant oxidation-dependent fluorogenic dye, CM-H$_2$DCFDA (Invitrogen). SU-DHL-1 cells were plated at $2 \times 10^5$ cells/well onto 24-well plates in phenol red-free RPMI. The cells were washed and resuspended in PBS containing 10 µM CM-H$_2$DCFDA for 30 min in the dark and stimulated with 1 mg/ml Hb, with or without pretreatment with 0.1 µg/ml anti-CD163. The fluorescence of the dye at 495 nm was measured using a microplate reader (Biotek).

### 2.2.1.3 Mitochondrial ROS detection using MitoSOX Red dye

The ROS generated in the mitochondria of cells was measured using the cell permeant mitochondrial specific fluorogenic dye, MitoSOX Red (Invitrogen). The cells were washed and resuspended in PBS containing 5 µM MitoSOX Red for 10 min at 37°C in the dark. The cells were then washed thrice and

resuspended in PBS. The fluorescence of the dye was measured on a CyAn ADP flow cytometer (Dako).

## 2.2.2 Cloning of CD163 and expression in HEK293T cells

The full length human CD163 was amplified from the human primary monocyte cDNA using forward and reverse primers (CD163 FW and CD163 RW, refer List of primers) with Xho I and Hind III restriction sites and ligated into pcDNA3.1A (Invitrogen). The ligation mixture was then transformed into the *Escherichia coli* TOP10 competent cells and the positive colonies were screened by colony PCR. The positive clones were verified by DNA sequencing. The plasmid was transfected into HEK293T cells, grown overnight on 12-well plates (Nunc) at a density of $4 \times 10^5$ cells/well in complete DMEM. The cells were transfected using Turbofect (Fermentas) according to the manufacturer's instructions. Briefly, 2 μg of CD163-pcDNA3.1 or empty vector was mixed with 4 μl turbofect in 200 μl of serum-free DMEM, incubated for 30 min at room temperature and added to the cells. The expression of CD163 was verified at the protein level using Western blotting 24 h post transfection.

## 2.2.3 Cell viability and apoptosis assays

### 2.2.3.1 Cell Titer Blue viability assay

Cell viability was measured using the CellTiter-Blue viability assay kit (Promega) following the manufacturer's instructions. Briefly, HEK293T and HepG2 cells seeded overnight on 96-well plates were stimulated with Hb. Cell

Titer-Blue was added to each well, and fluorescence was measured (excitation 530 nm, emission 590 nm) after 4 h of incubation. The mean fluorescence of triplicate wells was calculated and plotted.

## 2.2.3.2 Annexin-V and propidium iodide assay

Staining of early apoptotic cells was performed using the Annexin-V-FITC Apoptosis Detection kit (eBioscience) and propidium iodide (PI) viability staining solutions (eBioscience) according to the manufacturer's instructions. Briefly, primary monocytes were stimulated with 1 mg/ml of native Hb or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163. The cells were then washed successively with PBS and 1X binding buffer and resuspended in binding buffer at a density of 1 x $10^6$ cells/ml. The cells were incubated with FITC conjugated Annexin-V (20:1 (v/v)) for 15 min at room temperature and washed. PI was added at a dilution of 1:20 to the cell suspension and immediately analyzed on CyAn ADP flow cytometer (Dako). Annexin-V stains early apoptotic cells while PI stains late apoptotic and dead cells.

## 2.2.3.3 Mitochondrial membrane potential assay

The mitochondrial membrane potential in live cells was measured using the cell-permeant TMRE dye (Sigma). Briefly, cells were washed once with PBS and incubated with 100 nM TMRE in PBS for 20 min at 37$^o$C. The cells were then washed once and resuspended in PBS and analyzed immediately on a CyAn ADP flow cytometer (Dako). Intact mitochondria would retain the

TMRE dye and detected by its fluorescence signal. Cells treated with 10 μM Carbonylcyanide m-chlorophenylhydrazone (CCCP, a mitochondrial uncoupling agent) and 100 μM hydrogen peroxide ($H_2O_2$) were used as positive controls to reduce mitochondrial potential.

### 2.2.3.4 Caspase activity assays

The activities of caspase-3 and -9 were measured by resuspending the cell pellets in 30 μl of phiphilux-$G_2D_2$ and caspalux 9-$M_2D_2$ substrates, respectively (OncoImmunin) and incubating for 40 min at 37°C. After washing in FACS buffer, the cells were analyzed at λex 552 nm/ λem 580 nm.

### 2.2.4 Flow Cytometry Analysis
### Surface CD163 expression on monocytes

$2 \times 10^6$ SU-DHL-1 cells were washed twice with PBS and fixed in 4% (w/v) paraformaldehyde for 15 min. The cells were then blocked with 2% BSA for 30 min and washed once with PBS (pH 7.4). Subsequently, the cells were sequentially stained with primary antibody mixture [goat anti-CD163 (1:100); mouse anti-PMCA ATPase (1:200)] and secondary antibody mixture [(Alexa 488-conjugated donkey anti-goat (1:200); Alexa 546-conjugated donkey anti-mouse (1:500) (Invitrogen)] with two washes following primary antibody incubation. Then the cells were washed thrice with PBS and 10, 000 cells were acquired and analyzed on the CyAn ADP flow cytometer (Dako). The polyclonal anti-CD163 detects the full-length protein. The fluorescence intensity values for CD163 were normalized against the corresponding values for the membrane housekeeping control protein, PMCA-ATPase.

**Assay for Hb endocytosis by CD163⁺ U937-derived macrophages**

$10^6$ U937-derived macrophages were incubated with 0.5 mg/ml Hb for 45 min and excess Hb was removed by washing twice with PBS. The cells were fixed in 4% paraformaldehyde (PFA) for 15 min and permeabilized using 0.5% Tween-20 in PBS for 15 min and then blocked with 3% BSA for 30 min. The endocytosed Hb was stained using anti-Hb and Alexa-488-conjugated secondary antibody and analyzed on the FITC channel ($\lambda_{ex}$: 490 nm $\lambda_{em}$: 525nm) on the Cyan ADP flow cytometer (Dako).

**Measurement of knockdown efficiency of FcγRs in primary monocytes**

Human primary monocytes were nucleofected (refer section 2.2.7) with target specific siRNA pool for FcγR1 (CD64), FcγRII (CD32) and FcγRIII (CD16) (refer section 2.2.7). To measure the knockdown efficiency, $1 \times 10^6$ cells were harvested 48 h post-nucleofection, washed twice with PBS and fixed in 4% (w/v) paraformaldehyde for 15 min. The cells were then blocked with 2% BSA for 30 min and washed once with PBS (pH 7.4). Subsequently, the cells were sequentially stained with 1 µg primary mouse anti-human CD64/CD32/CD16 antibodies (1:100) and Alexa 488-conjugated donkey anti-mouse antibody (1:500) (Invitrogen) with two washes following primary antibody incubation. Then the cells were washed thrice with PBS, and 10,000 cells were acquired and analyzed on the CyAn ADP flow cytometer (Dako).

**Binding of sCD163:FITC-Hb:IgG complex to monocytes**

Wild type, CD64-, CD32- and CD16-knocked down primary human monocytes were incubated with sCD163:FITC-Hb:IgG complex (0.5-2 µM) for 30 min. The cells were then washed thrice with PBS and endocytosis of the complex was quantitated using the CyAn ADP flow cytometer on the FITC channel. Data are representative of three independent experiments using primary monocytes from a single healthy donor. BSA treated cells served as negative control.

**Measurement of signaling molecules**

$3x10^6$ U937-derived macrophages were washed with PBS and fixed in 4% (w/v) paraformaldehyde for 15 min. The cells were then washed and permeabilized with 0.5% Tween-20 in PBS (PBST) for 15 min. Subsequently, the cells were blocked with 2% BSA for 30 min and washed once with PBS (pH 7.4). The cells were sequentially stained with primary rabbit antibody (anti-pERK (1:100)/ anti-HO-1 (1:200)/ anti-NRAMP-1 (1:200)/ anti-Bax (1:50) and Alexa-488 conjugated secondary antibody (1:400) (chicken anti-rabbit; Invitrogen) followed by two washes after incubation with the primary antibody. The cells were then washed four times with PBS, and 10, 000 cells in the gated region were acquired and analyzed on the CyAn ADP flow cytometer (Dako). Cells stained with secondary antibody alone served as negative control.

The protein level of HO-1 was measured in HMVEC cells stimulated with increasing doses of Hb alone or preformed sCD163:Hb:IgG complex for 6 h and sequentially stained with rabbit anti-HO-1 (Cell Signaling) and Alexa-

488 conjugated chicken anti-rabbit (Invitrogen) as described above. 10, 000

cells in the gated region were acquired and analyzed on the CyAn ADP flow

cytometer (Dako). Cells treated with hemin, which is the substrate of HO-1,

served as positive control.

## 2.2.5 ELISA

### Soluble CD163-specific ELISA

SU-DHL-1 cells, plated at a density of $2 \times 10^6$ cells/well in 24-well plates,

were stimulated with 1 mg/ml Hb over a time course of up to 60 min. This

concentration was chosen in view of its pathophysiological relevance

(Philippidis et al, 2004). The cells were pelleted at 300xg for 15 min at $22^{\circ}C$,

and the concentration of sCD163 in the cell culture supernatants was measured

using human CD163 ELISA kit (Quantikine, R&D Systems).

### Interaction between sCD163, Hb and IgG

$1 \times 10^8$ freshly isolated human primary monocytes were washed twice with

PBS, pH 7.4 and stimulated with $10^{-8}$ M PMA for 2 h at $37^{\circ}C$. sCD163 was

isolated from the culture supernatants (refer section 2.1.5). The concentration

of the affinity-purified sCD163 was determined using CD163-specific ELISA.

sCD163 (0.1 µg/ml) in 100 µl coating buffer (50 mM sodium

carbonate/bicarbonate, pH 9.6) was immobilized onto 96-well microplates

(NUNC) by incubating overnight at $4^{\circ}C$. Increasing concentrations of Hb in

PBS was added and the reaction was incubated for 2 h at $37^{\circ}C$. Following

three washes, the bound Hb was detected using 1:1000 mouse anti-Hb (Santa

Cruz) and 1:2000 goat anti-mouse-HRP (Dako). The optical density (OD) at 405 nm was read using the microplate reader.

To test the interaction between sCD163:Hb complex and IgG, anti-IgG in the coating buffer was immobilized onto 96-well microplates as described above and used to capture IgG from human serum. Increasing concentrations of the preformed sCD163:FITC-Hb complex was added and incubated for 2 h at room temperature. Following five washes, the FITC fluorescence was read using the microplate reader. sCD163:FITC-BSA served as the negative control. The readings were subtracted from the values obtained with FITC-Hb alone.

**Subcellular tracking of biotinylated sCD163**

Primary human monocytes were incubated with either bitoin-sCD163 (refer section 2.1.8) alone or as a complex with Hb and IgG over a time course of up to 90 min at room temperature. The membrane and cytosolic fractions isolated from cells were captured on anti-CD163 coated 96-well microplates for 2 h at room temperature. The biotin-labeled protein bound to the plate was detected by HRP-Streptavidin Conjugate (ZyMax$^{TM}$ Grade, Invitrogen). ABTS substrate enabled the detection of the HRP conjugate and OD at 450 nm was read using the microplate reader. Three washes with PBST were carried out between incubations.

**Quantification of cytokines**

The levels of TNF-$\alpha$, IL-8 and IL-10 in the culture supernatants were measured using commercially available kits (OptEIA Human TNF-$\alpha$, IL-8 and IL-10 ELISA Kits, BD Biosciences), following the manufacturer's instructions.

**2.2.6 Fluorescence microscopy**

**2.2.6.1 Immunostaining of CD163 on monocytes**

SU-DHL-1 cells or primary monocytes were seeded at a density of 2 x $10^5$ cells/well onto poly-lysine (Sigma) coated coverslips and cultured overnight. The cells were then incubated with 1 mg/ml of native or activated Hb for up to 60 min. Subsequently, the cells were fixed using 4% (w/v) paraformaldehyde for 10 min, blocked with 1% BSA in PBS and incubated with a mixture of primary antibodies containing goat polyclonal anti-CD163 (1:200) (R&D Systems) for 60 min at room temperature. Following three washes with PBS, pH 7.4, the cells were incubated with secondary NL-557 conjugated donkey anti-goat antibody (1:200). Following three washes with PBS, the coverslips were mounted on a slide along with the ProLong Gold antifade-mounting reagent containing DAPI (Invitrogen). Images were acquired using Axio Observer Z1 fluorescence microscope (Zeiss) under 32x air objective.

## 2.2.6.2 Subcellular tracking of sCD163 and Hb in monocyte-endothelial co-culture

Confluent HMVEC cells grown on coverslips were washed twice with PBS and incubated with freshly isolated primary monocytes or THP-1 cells at a ratio of 1:1 in PBS with or without Hb treatment for 45 min. Subsequently, the cells were fixed using 4% (w/v) paraformaldehyde for 10 min, blocked with 1% BSA in PBS and incubated with a mixture of primary antibodies containing goat polyclonal anti-CD163 (1:200) (R&D Systems) and rabbit anti-Hb (1:500) (Sigma), for 60 min at room temperature. Following three washes with PBS, pH 7.4, the cells were incubated with secondary antibody mixture containing NL-557 conjugated donkey anti-goat (1:200) and Alexa-488 conjugated chicken anti-rabbit (1:400). The cells were then washed thrice with PBS and mounted on a slide along with the ProLong Gold antifade-mounting reagent containing DAPI (Invitrogen). Confocal imaging of the cells was performed on an LSM 510 META microscope (Zeiss) under 100x oil immersion objective using the LSM 510 software.

## 2.2.6.3 Live-imaging of intracellular ROS production in cells

Real-time imaging of intracellular ROS production by Hb was monitored by stimulating CM-H$_2$DCFDA-loaded CD163$^+$ primary monocytes with 0.1 mg/ml of native or activated Hb with or without pretreatment with 0.1 µg/ml anti-CD163 for up to 200 s on the LSM510 META confocal microscope (Zeiss). N-acetyl cysteine (NAC), a ROS scavenger and human serum albumin (HSA) treated cells served as negative controls. Control CD163$^-$ Jurkat cells were used to show the specific effect of CD163 on the Hb-ROS production.

**2.2.6.4 Live-imaging of Hb-induced monocyte-endothelial cell interaction**

To monitor the Hb-induced monocyte-endothelial interactions, human primary monocytes and HMVEC cells were co-cultured on sterile coverslip-bottom dishes (BD Biosciences). The cells were then washed gently with PBS and stimulated with 1 μg Hb in the presence or absence of IgG for up to 20 min and images were taken at a 30 s interval on the Axio Observer Z1 fluorescence microscope (Zeiss) under 32x air objective under the phase contrast mode.

**2.2.6.5 Mitochondrial staining**

Mitotracker Orange (Invitrogen), a cell-permeant mitochondrial labeling dye was used to stain mitochondria in U937 derived macrophages. Mitotracker Orange was chosen to allow for co-staining of bacteria with FITC and nucleus (DAPI). Briefly, treated cells were washed once with PBS. Subsequently, the cells were incubated with 20 nM Mitotracker Orange for 30 min at $37^{o}$C and washed twice in PBS for 5 min. The cells were then fixed and mounted on clean slides with ProLong Gold anti-fade mounting medium (Invitrogen) containing DAPI. Images were acquired using the LSM 510 META confocal microscope (Zeiss) under the 100x oil immersion objective.

**2.2.6.6 Staining of intracellular *S. aureus* and *S. typhimurium***

In order to track the entry of *S. aureus* or *S. typhimurium* into the macrophages, we stained the bacteria with BacLight Red Stain (Molecular Probes). The bacteria were cultured for infection using similar methods as mentioned previously (refer section 2.1.2) and resuspended at a concentration

of $10^9$ cells/ml. The bacteria were then stained for 15 min at room temperature using the BacLight Red Stain at a recommended concentration of 0.5 μM. The excess stain was then washed away and the bacteria were used for infection and processed for viewing under LSM 510 META confocal microscope (Zeiss) at 100x oil immersion objective. Z-stack images were acquired at 0.48 μm interval above and below the focal plane to ensure the intracellular localization of the bacteria.

## 2.2.7 siRNA mediated knockdown of FcγR in primary monocytes

To validate the role of FcγR in the uptake of sCD163: Hb: IgG complex, we silenced the three isotypes of FcγR - FcγR1 (CD64), FcγRII (CD32) and FcγRIII (CD16). The CD64 targeting siRNA pool was obtained from Dharmacon (Thermo Scientific), while the CD32 and CD16 siRNA duplexes were from OriGene Technologies. 2.5 x $10^6$ primary monocytes were nucleofected with 2 μg siRNA pool using the Amaxa Nucleofector (human monocyte Nucleofector kit, Nucleofector program Y-001). The oligonucleotide sequence of the siRNA pool used to knockdown the FcγR types in primary monocytes are shown in **Table 2.1**. Scrambled siRNA pool was used as the negative control. Cells were harvested 48 h post-nucleofection. The efficiency of knockdown was analyzed at the protein level by flow cytometry.

**Table 2.1**: siRNA pool used to knockdown human FCGRI, FCGRII and FCGRIII

| Gene | siRNA sequence (5'-3') |
|---|---|
| FCGR1<br><br>(CD64) | AAACAAAGUUGCUCUUGCA<br>GGAAAUGUCCUUAAGCGCA<br>GGAACACAUCCUCUGAAUA<br>GAGAAGACUCUGGGUUAUA |
| FCGRII<br><br>(CD32) | rArGrArArCrArArArGrArGrCrCrCrArArUrUrArCrCrArGAA<br>rGrArUrGrUrArGrCrArArArCrArUrGrArGrArArArCrGrCrUTA<br>rGrArArUrUrArGrArArGrArGrGrGrUrGrArGrGrGrArUrCrUrGrGTA |
| FCGRIII<br><br>(CD16) | rGrCrUrUrCrGrCrUrGrArGrGrUrUrArArGrGrUrUrArUrGrArArAAC<br>rCrGrArUrGrArGrGrUrCrCrUrCrUrUrArArArUrGrCrUrArGrGAG<br>rArGrArArArUrArGrCrArGrGrGrUrArGrUrCrCrArGrGrGrArUAG |

## 2.2.8 Protein-Protein Interaction Analysis

## 2.2.8.1 Surface Plasmon Resonance

**Principle**

Surface plasmon resonance (SPR) was used to determine the real-time binding kinetics between IgG, Hb and sCD163 using a BIAcore 2000 instrument (BIAcore, Uppsala, Sweden). SPR is a label-free technique and capable of measuring real-time quantitative binding affinities using relatively small quantities of the analytes. SPR uses an optical method to measure the change in refractive index of the medium in close vicinity of a metal surface when analyte molecules are flown over receptor molecules immobilized on a sensor chip (Besenicar et al, 2006).

The surface is typically a thin film of gold on a glass support and forms the base of the flow cell through which small volume (~100 nl) of the analyte molecule is passed continuously (Patching, 2013). Polarized light from a laser source is directed to the bottom surface of the gold-film where surface plasmons are generated at the critical angle of the incident light. Interaction between the analyte and the immobilized receptor changes the refractive index at the surface of gold film and is measured by increase in the response units (RU) (1 RU equals shift in critical angle by $10^{-4}$ deg). When analyte molecules are flown over the receptor immobilized chip surface, interaction leads to an initial association phase during which the binding sites become occupied and the rate of association ($k_{on}$) can be measured by this curve (**Figure 2.1**). When steady state is reached, the maximum RU value can be used to estimate the binding affinity ($K_D$). During the dissociation phase, receptor binding sites become unoccupied and the shape of this curve can be used to calculate the dissociation constant ($k_{off}$). The surface can then be regenerated for a new round of analysis.

Binding models are used to fit the experimental data over a series of concentrations. The accuracy of fit between experimental data and binding models are judged by the extent of overlap between the experimental and fitting curves. The chi-square value represents the sum of squared differences between the experimental data and fitted data at each data point.
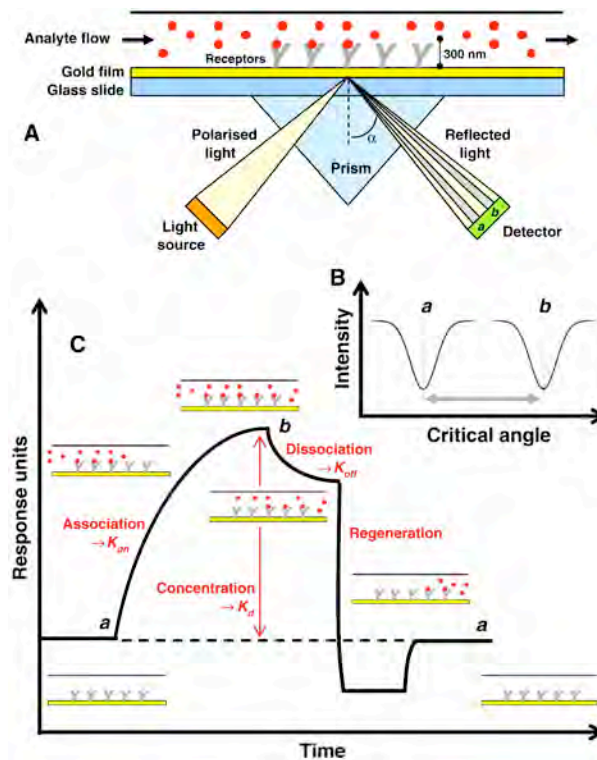
**Figure 2.1: Schematic illustration of SPR assay.** (**A**) Analyte molecules are flown over the receptor immobilized on the chip surface. Polarized light is directed through a prism to the bottom surface of the gold film where surface plasmons are generated at the critical angle of incident light. (**B**) Change in the critical angle of the incident light from a to b when the analyte binds to the receptor. (**C**) A typical SPR sensogram featuring the association phase and the dissociation phase. Figure adapted from (Patching, 2013) with permission from Elsevier publishers.

**Assay**

The real time biointeraction between IgG, Hb and sCD163 was analyzed by surface plasmon resonance using a Biacore 2000 instrument (Biacore International AB, Uppsala, Sweden). IgG was immobilized on a CM5 chip by amine coupling according to the manufacturer's instructions. Increasing doses of Hb at 13-51 µg/ml, was injected over the IgG-immobilized chip in running buffer of 50 mM Tris, 145 mM NaCl with 2 mM calcium, pH 7.4, at a flow

rate of 30 µl/min. Anti-Hb at 5-20 nM was injected to verify the specificity of interaction between Hb and IgG. sCD163 was buffer-exchanged to the same running buffer using Vivaspin columns (Sartorius Stedim Biotech, France) and 50 µl sCD163 (2.5-10 ng/ml) was injected over the bound Hb. The dissociation phase was carried out for 180 s at the same flow rate. Regeneration of the chip surface was performed by injection of 0.1 M NaOH until baseline was restored. The binding affinities were calculated using BIAevaluation software, version 4.1 applying the drifting baseline model assuming 1:1 interaction model. Dashed lines represent the curve fitting. Response units were subtracted from BSA-N-acetylglucosamine immobilized reference flow cell (negative control).

### 2.2.8.2 Co-immunoprecipitation (Co-IP)

To test for interaction between sCD163 and Hb, 10 µg of anti-CD163 (R&D Systems) in TBS, pH 7.5 was conjugated to Protein-A Sepharose beads (GE Healthcare Life Sciences) overnight at 4$^{\circ}$C. Unbound antibody was removed by washing twice with TBS and the bound anti-CD163 was cross-linked to sepharose by incubating for 60 min in cross-linking buffer containing 50 mM dimethyl pimelimidate (Sigma) and 200 mM triethanolamine, pH 8.9. The Sepharose beads were blocked using 100 mM ethanolamine, and then incubated with 5 µg sCD163 and 5 µg subtilisin-A treated Hb in 500 µl of binding buffer (TBS, pH 7.4) for 60 min at room temperature with two washes between each binding step. Subsequently, after three washes, the bound proteins were eluted with 2.5% acetic acid, into tubes containing neutralization buffer of 1 M Tris-HCl, pH 12.0.

To test for interaction between IgG, Hb and sCD163, we incubated protein A-Sepharose beads with 5 µg IgG (affinity-purified from human serum) in 500 µl TBS at room temperature for 60 min. The unbound antibody was removed by washing twice in TBS. The beads were incubated with 5 µg subtilisin A-treated Hb and 5 µg sCD163 in 500 µl of binding buffer (TBS, pH 7.4) for 60 min at room temperature with two washes between each binding step. Subsequently, after three washes, the bound proteins were eluted using 2.5% acetic acid into tubes containing 1 M Tris-HCl, pH 12.0.

### 2.2.8.3 Proximity ligation assay (PLA)

### Principle

The proximity ligation assay (PLA) is used for specific *in situ* detection of native-protein complexes in cells and subcellular compartments. The PLA method depends on the dual proximity binding by a pair of detection reagents to generate amplifiable DNA strands, which then serve as surrogate markers for the detected proteins (Fredriksson et al, 2002). Briefly, a pair of oligonucleotide labeled secondary antibodies (PLA probes-MINUS and PLUS) is brought to proximity by the interacting proteins bound to the primary antibodies (**Figure 2.2**). The ligation solution consisting of two short oligonucleotides and ligase is added. The oligonucleotides will then ligate to the PLA probes and join to form a closed circle, which is amplified by the rolling circle amplification using a polymerase (Soderberg et al, 2008). The oligonucleotide arm of one PLA probes acts as a primer for the rolling circle amplification reaction using the ligated circle as a template and generates a concatemeric product. Fluorescently labeled oligonucleotides are then added

which hybridize to the amplified product. The signal, which is representative of a single pair of interacting proteins, is visible as a distinct fluorescent dot and analyzed by fluorescence microscopy.



|     **Binding of**     | **Circularization and** | **Rolling circle** |
| **proximity probes** | **ligation of connector** | **amplification** |
|                        | **oligonucleotides** | **and detection** |
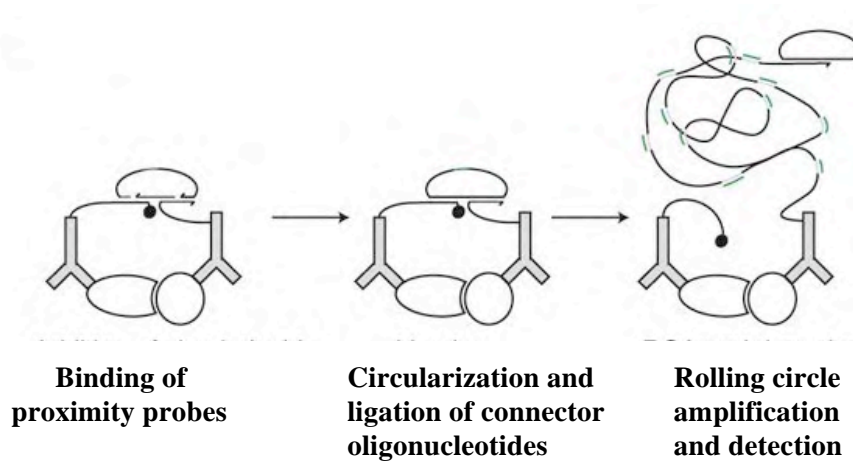
**Figure 2.2: Principle of the PLA assay.** Dual binding by a pair of PLA probes (antibodies with attached oligonucleotides) to a protein complex serves as a template for the hybridization of circularization oligonucleotides, which are then ligated into a circular DNA molecule. The circular DNA is amplified by rolling circle amplification (RCA) primed by a proximity probe and is covalently attached to the proximity probe. The RCA product can be detected by fluorescently labeled oligonucleotides. Figure adapted from (Soderberg et al, 2006) with permission from Nature publishing group.

**Assay**

To visualize specific protein–protein interaction between Hb and CD163 inside the monocytes *in situ,* PLA was performed using the Duolink detection 563 kit (Olink Biosciences, Uppsala, Sweden) following the manufacturer's instructions. Briefly, cells were plated at a density of 2 x $10^5$ cells onto 4-well chambered slides (Iwaki, Japan) coated with poly-lysine and left to attach

overnight. Cells were fixed in 4% (w/v) paraformaldehyde for 15 min and washed once with PBS. Then, the cells were incubated with 1 mg/ml of native or activated Hb for 30 min and washed once in PBS. The CD163:Hb complex was detected using goat anti-CD163 and rabbit anti-Hb primary antibodies. The corresponding probes of anti-rabbit PLUS, anti-goat MINUS were purchased from Olink Biosciences. The cells were then mounted with the ProLong Gold anti-fade reagent containing DAPI and imaged using the LSM 510 META confocal microscope (Carl Zeiss) under the 100x oil immersion objective.

## 2.2.9 Biotin switch assay for sCD163 palmitoylation

$1 \times 10^6$ human primary monocytes were incubated with sCD163 alone or sCD163:Hb or sCD163:Hb:IgG complex for up to 4 h. To verify the involvement of palmitoylation in the recycling of sCD163, the cells were pre-treated with 100 µM 2-bromopalmitate, a palmitoylation-inhibitor, or with 100 µM 2-hydroxy myristic acid, a control inhibitor, for 60 min at $37^0$ C. To confirm that sCD163 from the endocytosed sCD163:Hb:IgG complex is recycled to the membrane, the cells were pre-treated with 50 µM monensin, a recycling inhibitor, and 25 µg/ml chlorpromazine, an endocytosis inhibitor, for 60 min at $37^0$ C.

The cell pellet was suspended in 50 µl of lysis buffer (1X PBS, pH 7.4, 1X protease inhibitors, 1mM EDTA, 1% Triton X-100, 25 mM N-ethyl maleimide) for 1 h at $4^0$ C on a shaking platform. The lysate was then centrifuged at 14000xg for 20 min to remove insoluble material. The

supernatant, which contains proteins, was removed and the total protein concentration was measured using the protein assay kit (BioRad).

1 mg of total protein was resuspended in 200 µl of lysis buffer containing 0.5 % saponin (blocking agent) overnight at $4^0$ C on a shaking platform. The proteins were precipitated using methanol/chloroform method (3:1:4 ratio of methanol chloroform and water) and centrifuged at 10000xg for 30 min. The upper phase was discarded without disturbing the interphase and 4 volumes of methanol was added and incubated at $-20^0$ C for 20 min. It was then centrifuged for 20 min at 5000xg at $4^0$ C. The supernatant was discarded and the pellet was air-dried for 10 min at room temperature.

The air-dried pellet was resuspended in 200 µl of resuspension buffer (1X PBS, pH 7.4, 8 M urea and 2% SDS) in a sonicator-bath for 10 min and gently agitated at $37^0$ C until solubilized. The solution was then divided into two equal aliquots, out of which one portion was combined with 80 µl of 1 M fresh hydroxylamine, 1 mM EDTA, protease inhibitors and 100 µl fresh 4 mM biotin-HPDP (Pierce) and gently mixed for 1 h at room temperature. The remaining aliquot was treated identically except that hydroxylamine was replaced with 50 mM Tris pH 7.4. The proteins were precipitated using the methanol chloroform method as described earlier.

The pellet was resuspended in 20 µl resuspension buffer and 180 µl PBS containing 0.2% Triton X-100. An aliquot of 20 µl was saved to serve as loading control while the remaining sample was incubated with 15 µl of high capacity neutravidin-agarose beads (Pierce) for 1 h at room temperature on a shaking platform. The beads were then washed and the captured proteins were eluted in SDS-PAGE loading buffer at $95^0$ C for 5 min. The samples were then

analyzed by western blotting and probed for CD163 using goat anti-CD163 and HRP-conjugated rabbit anti-goat antibodies.

### 2.2.10 Heme-oxygenase-1 (HO-1) activity assay

HO-1 activity assay was performed as described earlier (Motterlini et al, 2000). Briefly, cells were harvested post stimulation with native or activated Hb and the cell pellet was suspended in ice–cold 100 mM phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$ and cells were disrupted by sonicating on ice for 30 s. The suspension was then centrifuged at 18000xg for 10 min at 4°C and the supernatant was added to 250 μl of a reaction mixture containing 0.1 mM NADPH, 1 mM NADP, 1 mM glucose-6-phosphate, 5 mU of glucose-6-phosphate dehydrogenase, 2 mg protein of rat liver cytosol (source of bilirubin reductase, prepared according to (Tenhunen et al, 1970)), 100 mM potassium phosphate buffer (pH 7.4) and 1 mg/ml hemin. The reaction was performed at 37°C in the dark for 1 h. The reaction was terminated on ice by addition of 1 ml chloroform. The extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40$ mM$^{-1}$ cm$^{-1}$). The HO-1 activity was expressed as micromoles of bilirubin/ milligram of protein /h. BSA treated cells served as negative control.

### 2.2.11 Tris-Tricine SDS-PAGE

Tris-Tricine-SDS-PAGE under reducing conditions was performed to resolve the fragments of Hb after its proteolytic cleavage by subtilisin A (Schagger,

2006). 5 μg of partially proteolysed Hb was loaded per well. The protein bands were detected by staining with Coomassie Blue.

## 2.2.12 SDS-PAGE and Western blotting

Cultured cells were harvested, pelleted, and protein extraction was performed in ice-cold RIPA lysis buffer (Cell Signaling Technology) containing 1 mM PMSF and 1X protease inhibitor cocktail (Sigma). Fifty microgram of total proteins was resolved by 10% SDS-PAGE under reducing condition and then electrotransferred to polyvinylidene difluoride membrane (BioRad) in Tris-glycine buffer with 20% methanol at 120 V for 90 min. The membrane was then blocked using 5% BSA in TBS (blocking buffer) for 60 min at room temperature. Following two washes with TBS, the membrane was probed overnight with the primary antibody diluted in blocking buffer. Following two washes to remove unbound antibody, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in blocking buffer for 1 h at room temperature on a shaking platform. Bands were visualized with Supersignal West Pico chemiluminescence substrate (Pierce).

## 2.2.13 Infection and infectivity assays

**Infection of macrophages with bacteria**

U937 derived macrophages were pre-treated with 0.5 mg/ml Hb or PBS for 45 min at 37°C in 5% $CO_2$ to enable endocytosis of Hb. The cells were washed once with PBS and then infected at multiplicity of infection (MOI) of 10 in

PBS containing 2% FBS for 30 min on a shaking platform. The extracellular bacteria were removed by spinning down cells at 140xg for 5 min and washing with PBS. The cells were then resuspended in PBS containing 2% FBS and maintained up to 4 h at 37°C. The supernatants were collected and stored at -80°C for cytokine measurements.

**Protease activity assay**

*S. aureus* and *S. typhimurium* were cultured overnight at 37°C in the protease-producing buffer. Secondary cultures were grown to exponential phase and the activity of the extracellular protease(s) in the culture supernatant was determined at 37°C by using 1% azocasein assay (Lee et al, 2005). Briefly, 50 μl of the culture supernatant was added to 300 μl of 1% (w/v) azocasein prepared in 50 mM Tris-HCl, pH 7.0 and incubated for 20 min at 37°C. Subsequently, 600 μl of ice-cold 10% (w/v) trichloroacetic acid was added with simultaneous vortexing. The samples were placed on ice for 10 min and centrifuged at 15000 rpm for 15 min. The quantity of acid-soluble material in the supernatant was measured by absorbance at 366 nm. One Unit (U) of protease activity was defined as an absorbance of 0.1 at 366 nm.

**Colony forming ability of intracellular bacteria**

To distinguish between extracellular and intracellular bacteria, the cells were infected *with S. aureus* or *S. typhimuirum* for 30 min after which the extracellular bacteria were removed by spinning down the infected cells at 140xg for 5 min and further washing twice with PBS. The number of viable intracellular bacteria was quantified by lysis of the infected U937

macrophages with 0.2% Triton X-100 for 10 min (Garzoni & Kelley, 2009). The cell lysate was plated at serial dilutions in sterile PBS on LB agar plates with the appropriate selection medium using the drop method. For each dilution, three drops of 10 µl each was applied onto a quadrant of the LB agar plate, with 3-30 colony forming unit (CFU) per drop considered as reliable (Herigstad et al, 2001). The plates were incubated at 37°C overnight.

## 2.2.14 PyroGene assay to test for LPS contamination

**Principle**

Endotoxin contamination in samples was measured using the PyroGene assay kit (Lonza Biosciences). The assay employs recombinant factor C (rFC), an endotoxin sensitive protein from the horseshoe crab (Ding & Ho, 2001). rFC is activated upon endotoxin binding, and the activated serine protease domain exposed then acts to cleave a synthetic substrate into a fluorogenic compound. The fluorescent product formed is detected using the excitation and emission wavelengths of 380 nm and 440 nm, respectively. The amount of endotoxin in the sample is proportional to the amount of fluorescent product formed and quantified from a standard curve.

**Quantification of endotoxin contamination in Hb**

The level of endotoxin present in the native Hb and subtilisin A-activated Hb was measured using the PyroGene assay. Various dilutions of Hb were prepared and PyroGene assay was performed in 96-well plates in 100 µl reaction volume according to the manufacturer's instructions.

## 2.3 Statistical analysis

Data represent means ± standard error of the mean (SEM) of three independent experiments conducted in triplicate each. Statistical significance was accepted when the $p$ value $< 0.05$ by paired two-tailed Student's $t$ test when comparing two groups, and ANOVA, when comparing more than two groups.

# CHAPTER 3
# RESULTS AND DISCUSSION

## 3.1 CD163 and IgG Co-defend against cytotoxic Hb in the absence of Hp

During severe hemolysis triggered by hemolytic infection and/ or inflammation, large amounts of extracellular Hb (0.5-2 mg/ml) released into the circulation, generates cytotoxic ROS and hence induces tissue damage. Previous studies in our lab have shown that microbial proteases proteolytically activate the intrinsic POX activity of Hb, which drives the production of damaging superoxide radicals. Hence the key issue is how the host innate immune system effectively tackles the cytotoxic effects of this "infection-activated" Hb. Although plasma Hp has been shown to play the key role in binding and reducing the redox reactivity of Hb during moderate hemolysis, Hp is quickly saturated and overwhelmed during severe hemolysis. Therefore, the residual toxic Hb in the plasma demands a quick and efficient detoxification and removal by alternative mechanisms independent of Hp.

We hypothesized that during severe hemolysis, the host relies on the frontline receptor, CD163 and other plasma proteins to protect against Hb-POX driven oxidative damage. We sought to decipher the direct effect of CD163 on the Hb-POX activity and the consequential cell-survival when Hp is depleted. Next, we wanted to elucidate the potential role of sCD163 and other plasma proteins towards regulating the redox active Hb. Further, the potential cross talk between monocytes and endothelial cells towards Hb clearance were also examined.

**3.1.1 Complex formation between CD163 and Hb**

**3.1.1.1** *In vitro* **infection model system**

To mimic an infection-mediated proteolysis of Hb, we used an *in vitro* infection model system consisting of the bacterial serine protease, subtilisin A and Hb in PBS (pH 7.4), incubated at $37^0$C to study the effects of proteolytically activated Hb. We used Tris-Tricine SDS-PAGE to visualize the extent of cleavage of Hb with increasing doses of subtilisin A and reaction time and concurrently measured the ROS production by both native and activated Hb. **Figure 3.1** shows that incubation of subtilisin A with Hb released POX-active fragments of <10 kDa from Hb (arrows), concomitant with increasing POX activity. Prolonged reaction time led to excessive proteolysis and loss of the 10-kDa Hb-POX fragments causing a decrease in the POX activity. The optimised dosage of subtilisin A (1.5 U) and reaction time (15 min) was henceforth used consistently in all future experiments to generate proteolytically activated Hb. The effect of activated Hb on monocytes was studied and compared with native Hb.
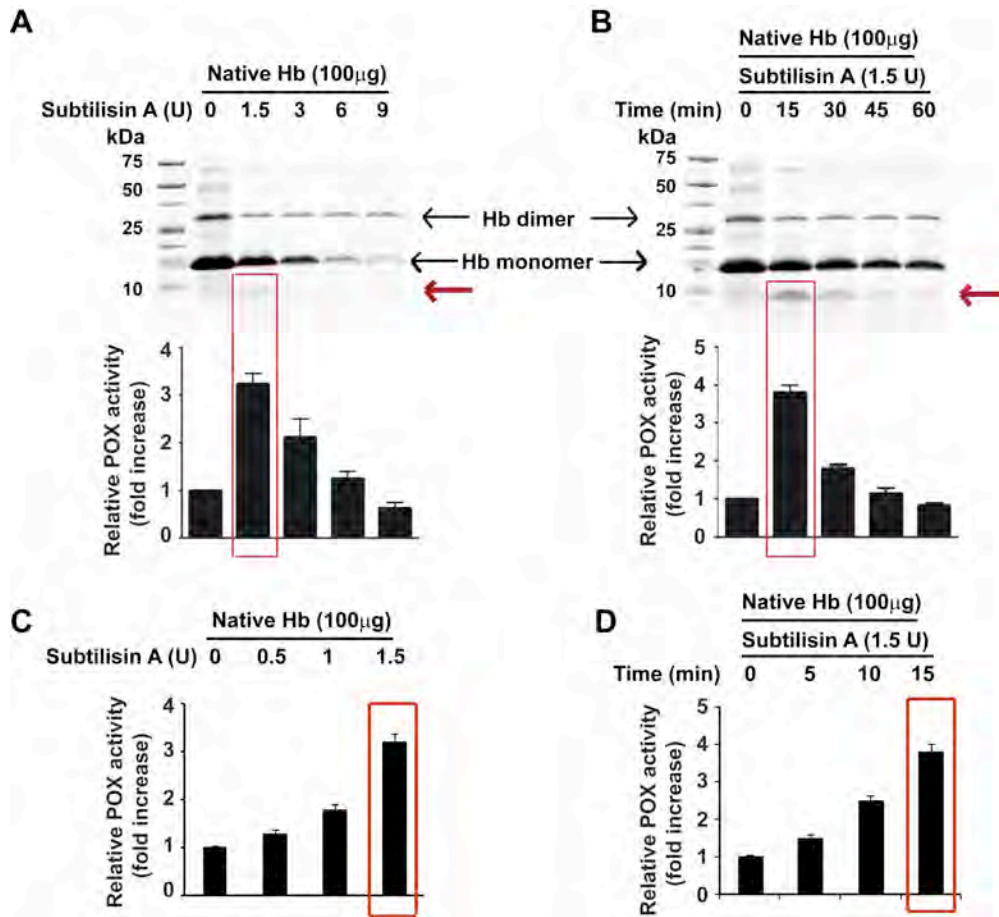
**Figure 3.1: Proteolytic activation of Hb by subtilisin A.** 100 μg Hb was incubated with: (**A**) increasing dosage of subtilisin A (0-9 U) for 15 min at 37°C and (**B**) subtilisin A (1.5 U) for a time course (0-60 min). Tris-Tricine SDS-PAGE (10%) gel was used to resolve the partially proteolysed Hb and the bands were stained with Coomassie blue. **Bottom panels** - The resulting Hb-POX activity was measured by using CLA-CL assay and expressed as fold-increase over that of the native Hb. Partial proteolysis of Hb is indicated by the appearance of bands below 10 kDa (red arrows), which is coincident with the accompanying rise in POX activity (red box). Hb-POX activity upon incubating 100 μg Hb with: (**C**) increasing dosage of subtilisin A (0-1.5 U) for 15 min at 37°C and (**D**) subtilisin A (1.5 U) for a time course (0-15 min).

**3.1.1.2 Hb associates with CD163 on monocytes**

It has been reported that CD163 binds to Hb in the absence of Hp, although with lower affinity compared to Hb:Hp complex (Schaer et al, 2006). Here, we investigated whether the proteolytically activated Hb, which is generated by the action of microbial proteases, could also be recognized by CD163 on the monocytes. CD163 and Hb were tracked by immunofluorescence microscopy in human primary monocytes. Freshly isolated human primary monocytes were stimulated with 1 mg/ml of native or activated Hb for 30 min and dual immunofluorescent staining for CD163 and Hb was performed following cell permeabilization.

Figure **3.2A** shows that both native and activated Hb were endocytosed by CD163, showing colocalization in monocytes. BSA-treated cells served as negative control to show the specific recognition of Hb by CD163. To further confirm the *in situ* interaction between CD163 and Hb, we performed the PLA assay (refer section 2.2.8.3). The PLA signals (red dots) are representative of the paired interactions between CD163 and Hb (**Figure 3.2B**), which was in accordance with the immuno-colocalization study. Cells in which the primary antibody was excluded for detection showed no positive signals for interaction. Cells treated with the control protein, BSA (denoted as "Unt") did not show any red-dots indicating the specificity of interaction between CD163 and Hb. Taken together the above results suggest that CD163 acts as a scavenger receptor for both the native and proteolytically activated Hb under severe hemolytic conditions.

### 3.1.2 CD163 directly detoxifies Hb and rescues cells from Hb-induced apoptosis

### 3.1.2.1 Effect of CD163 expression in HEK293T cells

To determine whether CD163 affects the Hb-POX activity, we transfected CD163 into HEK293T cells (CD163⁻) and then measured the POX activity of Hb upon incubation of the CD163⁺ HEK293T cells or mock-transfected control cells with activated Hb using the CLA assay (refer section 2.2.1.1). **Figure 3.3** (box) shows that within 10 min, the CD163⁺ HEK293T cells had reduced the POX activity by ~80% whereas the control cells were unresponsive, suggesting that the CD163 effectively blocked the Hb from producing $O_2^{\cdot-}$. The inhibition of Hb-POX activity by CD163⁺ HEK293T was also dose-dependent.
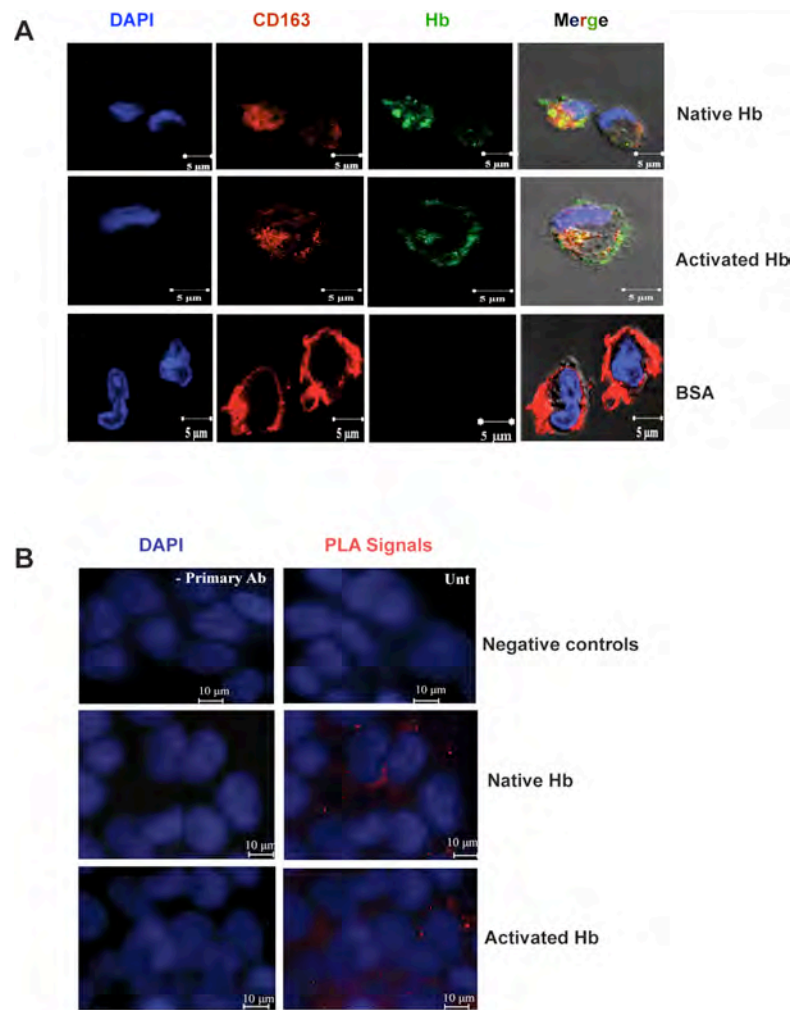
**Figure 3.2: CD163 endocytosed both native and proteolytically activated Hb.** (**A**) Co-localization of CD163 and Hb in primary human monocytes. Cells were incubated with native or activated Hb (1 mg/ml) for 30 min, fixed and stained with primary and secondary antibodies. Images were acquired using the LSM510 META confocal microscope. Scale bars, 5 µm. (**B**) Cells were cultured on poly-lysine coated chambered slides and incubated with native or activated Hb (1 mg/ml) for 30 min. The interaction between CD163 and Hb in the cells was confirmed using the proximity ligation assay (PLA). PLA was performed as per the manufacturer's instructions using anti-goat minus and anti-rabbit plus probes. "Unt" refers to cells treated with the control protein, BSA. Scale bars, 10 µm.
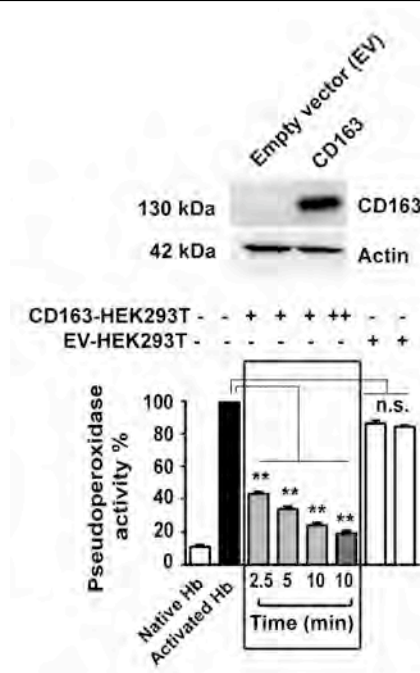
**Figure 3.3: CD163 inhibits Hb-POX activity. Top panel**- Western blotting to confirm the expression of CD163 in CD163-transfected HEK293T cells. **Bottom panel**- Hb-POX activity was measured over time of incubation of subtilisin A-activated Hb with $2 \times 10^5$ CD163$^+$ HEK293T cells or empty vector (EV) only transfected controls. Progressive decrease in Hb-POX activity was observed with time and dose (box). ++ denotes higher dose of CD163$^+$ HEK293T cells ($1 \times 10^6$ cells). ** represents p<0.005; n.s. denotes not significant.

## 3.1.2.2 Effect of monocyte CD163 on Hb-POX activity *in situ*

To test whether *in situ* membrane-associated CD163 (mCD163) directly inhibits Hb-POX activity, we added increasing doses of the native SU-DHL-1 membrane extract from CD163$^+$ SU-DHL-1 cells to Hb. The native membrane extract from the SU-DHL-1 cells had CD163, while the control HEK293T cells lacked CD163 (**Figure 3.4 A,** top panel). We found that the Hb-POX activity diminished dose-dependently of the membrane extract, both in the presence and absence of Hp. Upon incubation with 50 µg SU-DHL-1 membrane extract for 15 min, the POX activity of native and activated Hb was reduced by 60% and 80% respectively **(Figure 3.4 A, B)**.

71
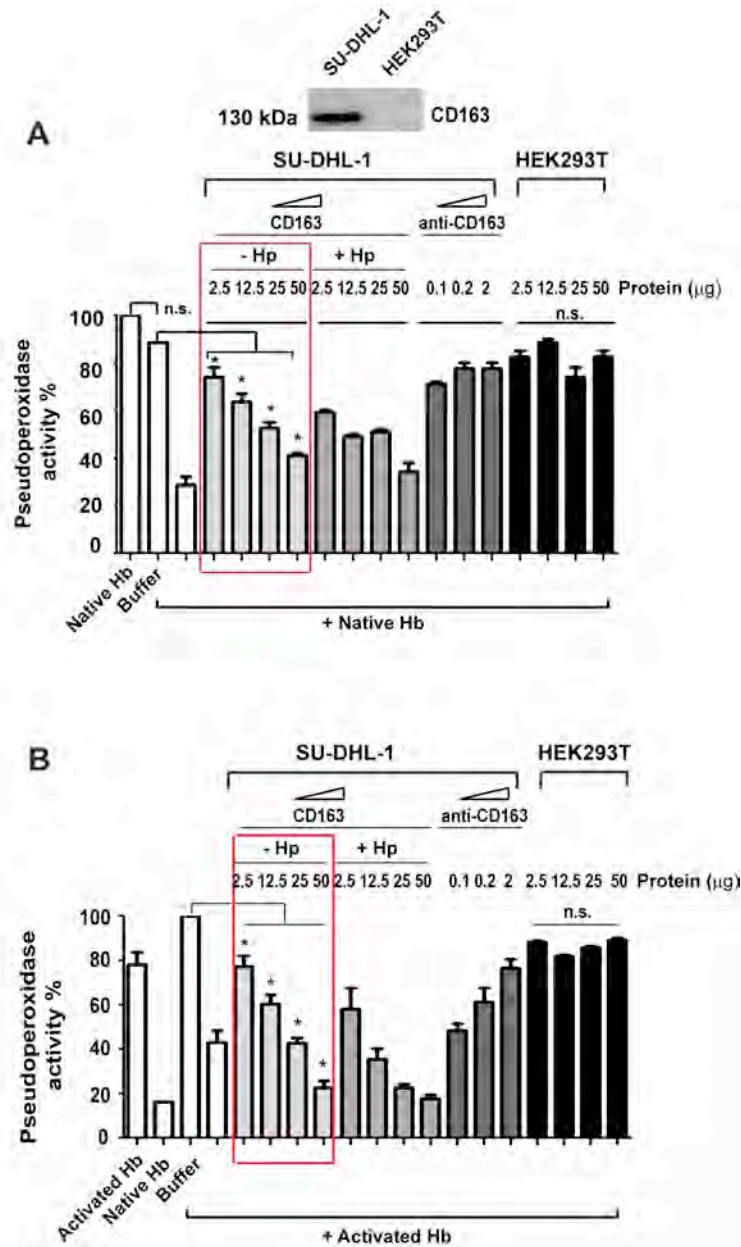
**Figure 3.4: Monocyte CD163 attenuates Hb-POX activity *in situ*.** The pseudoperoxidase (POX) activity of: (**A**) 10 μg native Hb and (**B**) 10 μg activated Hb upon incubation for 15 min with increasing doses of the native membrane protein extracts of CD163+ SU-DHL-1 or CD163- HEK293T cells with or without pretreatment with anti-CD163. Haptoglobin (Hp1-1 isoform) was used as a positive control. Red box indicates progressive decrease in Hb-POX activity upon addition of increasing doses of the SU-DHL-1 membrane extract containing CD163. * represents p<0.05; n.s. denotes not significant.

Incubation of native and activated Hb with the membrane extract from the control CD163⁻ HEK293T cells did not cause any significant reduction in the POX activity, implying the significance of CD163 in dampening Hb-POX activity. Addition of Hp, a positive control (Hp1-1 isoform (Sadrzadeh & Bozorgmehr, 2004)) further reduced the POX activity dose-dependently of the membrane extract. Importantly, when the SU-DHL-1 membrane extract was pre-incubated with anti-CD163, the inhibition of the POX activity was abrogated dose-dependently of anti-CD163, suggesting that mCD163 directly and specifically attenuates Hb-POX activity.

### 3.1.2.3 CD163 attenuates Hb-induced intracellular ROS production

Having shown that CD163 directly inhibits Hb-POX activity *in vitro*, we then measured the dynamics of ROS production within the CD163⁺ SU-DHL-1 cells when challenged with Hb, with or without pre-incubation with anti-CD163. **Figure 3.5 A, left panel** shows that by 300 s, activated Hb induced ~75% higher ROS production than native Hb. In the presence of functional CD163, the Hb-generated intracellular ROS was halved compared to when CD163 was pre-blocked using an antibody (**Figure 3.5 A, right panel**). *Ex vivo* real time quenching activity of Hb-POX by CD163 was also observed in human primary monocytes by live-cell imaging (**Figure 3.5 B and Videos 1-7**). Cells treated with N-acetyl cysteine (NAC), a ROS scavenger and the control protein, human serum albumin (HSA) served as negative control. Control cells (Jurkat, HEK293T and HepG2) devoid of CD163, succumbed to Hb, showing increased intracellular ROS and concomitant cell death (**Figure 3.5 B, C**), supporting the protective role of CD163 against the cytotoxic Hb.

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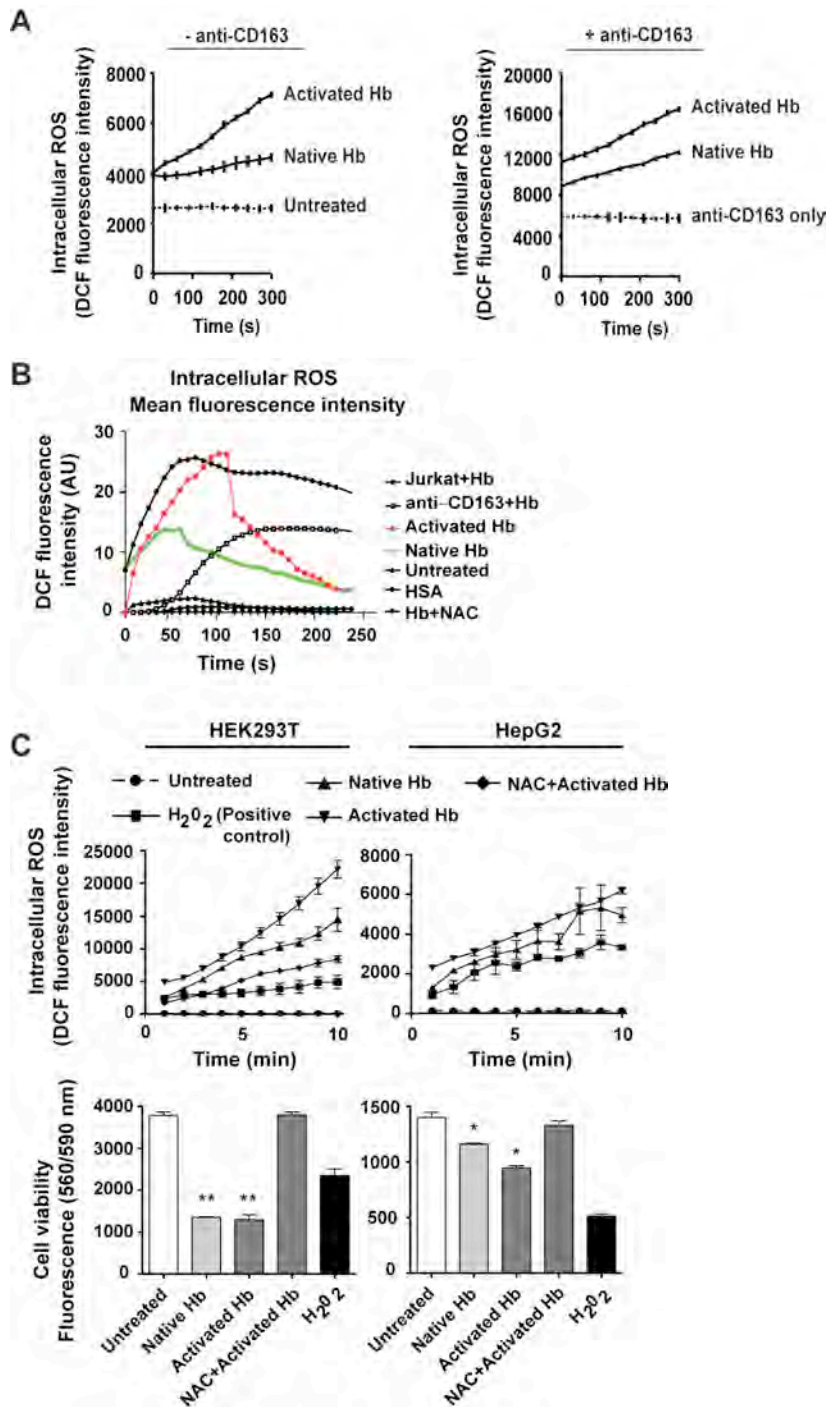
**Figure 3.5: CD163 protects cells from Hb-induced intracellular ROS.** (**A**) Intracellular ROS production in SU-DHL-1 cells incubated with 1 mg/ml of native Hb or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163. (**B**) Real-time production of intracellular ROS in human primary monocytes treated with 1 mg/ml of native or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163. HSA and NAC-treated cells served as negative controls. (**C**) Intracellular ROS and cell viability of CD163⁻ HEK293T and HepG2 cells stimulated with 1 mg/ml of native or activated Hb. $H_2O_2$, an inducer of ROS was used as positive control. Untreated in panels A, B and C represent cells treated with PBS only. ** indicates p<0.005; * indicates *p*< 0.05; n.s. denotes not significant.

74

### 3.1.2.4 CD163 suppresses Hb-induced apoptosis in monocytes

To demonstrate the biological significance of CD163-mediated scavenging and inhibition of Hb redox-reactivity, we examined the effect of accumulation of Hb-generated intracellular ROS on cell survival. In addition, we queried the consequence of blocking CD163 when Hb reaches concentrations as high as that of severe hemolysis during which Hp is depleted. We measured the dynamics of apoptosis when primary monocytes were challenged with Hb with or without blocking of CD163 using antibody. Flow cytometric analyses using Annexin-V FITC and propidium iodide consistently showed that the induction of apoptosis by native and activated Hb was time-dependent and by 4 h, activated Hb induced ~50% more apoptosis when CD163 was blocked (**Figure 3.6**). Notably, Hb-induced apoptosis was suppressed by the activity of fully functional CD163. Taken together, our findings suggest that CD163 could directly shield monocytes from Hb-POX induced cytotoxicity during a severe hemolysis.
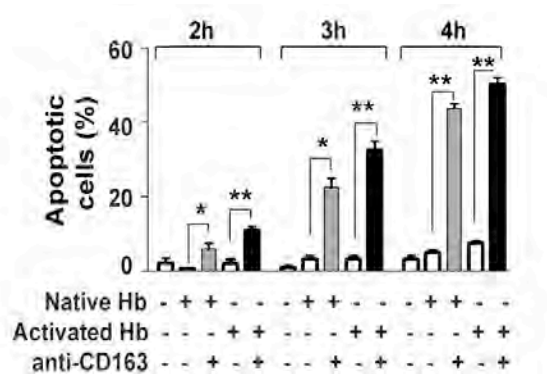


**Figure 3.6: CD163 protects monocytes from Hb-induced apoptosis.** Dynamics of apoptosis in primary monocytes stimulated with 1 mg/ml of native Hb or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163. The cells were stained with Annexin-V FITC and propidium iodide. * indicates $p < 0.05$; ** $p < 0.005$; n.s. denotes not significant.

75

### 3.1.3 Hb induces shedding of CD163 from monocyte membrane

Monocytes exposed to inflammatory stimuli are known to shed CD163 (Droste et al, 1999). To examine the effects of the highly inflammatory Hb-POX on mCD163 shedding, we stimulated SU-DHL-1 cells with 1 mg/ml of native or proteolytically-activated Hb and measured the density of mCD163 on the cells by flow cytometry. To quantiate the residential membrane-bound CD163 on the monocytes, the cells were not permeabilized during the antibody staining. We found that the level of mCD163 on the monocytes started to decline within 10 min of stimulation with native Hb, down to ~60 % by 1 h but recovered completely within 3-4 h (**Figure 3.7 A, B**). In contrast, activated Hb induced a more dramatic and steeper drop of mCD163 to ~30%, and the cells recovered only up to 50% of the mCD163 after 4 h. Control cells treated with subtilisin A alone did not shed CD163, implying the specificity of Hb-induced CD163 shedding. The Hb-mediated regulation of the level of mCD163 was specific since the housekeeping protein, plasma membrane calcium ATPase (PMCA ATPase), remained unaffected (Figure 3.7 B).
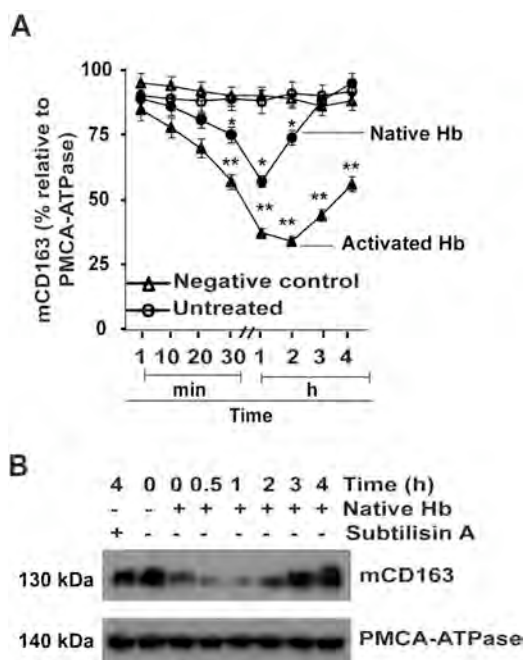


**Figure 3.7: Hb induced CD163 shedding from monocytes**. (**A**) Flow cytometry and (**B**) Western blot analysis of the resident mCD163 levels on SU-DHL-1 monocytes treated with 1 mg/ml of native or activated Hb. Subtilisin A alone-treated cells served as negative control. PMCA-ATPase, a housekeeping membrane protein was used as a loading control. Untreated refers to cells treated with PBS. * indicates $p < 0.05$; ** $p < 0.005$.

To corroborate the Hb-induced CD163 shedding from monocytes, we used immunofluorescence microscopy to analyze the resident level of CD163 on monocytes treated with native or activated Hb. To stain the membrane-associated CD163, the cells were not permeabilized during the antibody staining procedure. Consistent with flow cytometric analysis (Figure 3.7 A) and immunoblotting (Figure 3.7 B), immunofluorescence microscopy showed fewer CD163$^+$ cells at 60 min post-stimulation with native Hb (**Figure 3.8**). Activated Hb caused a further reduction in the number of CD163-stained cells. Cells treated with the control protein, BSA served as negative control.
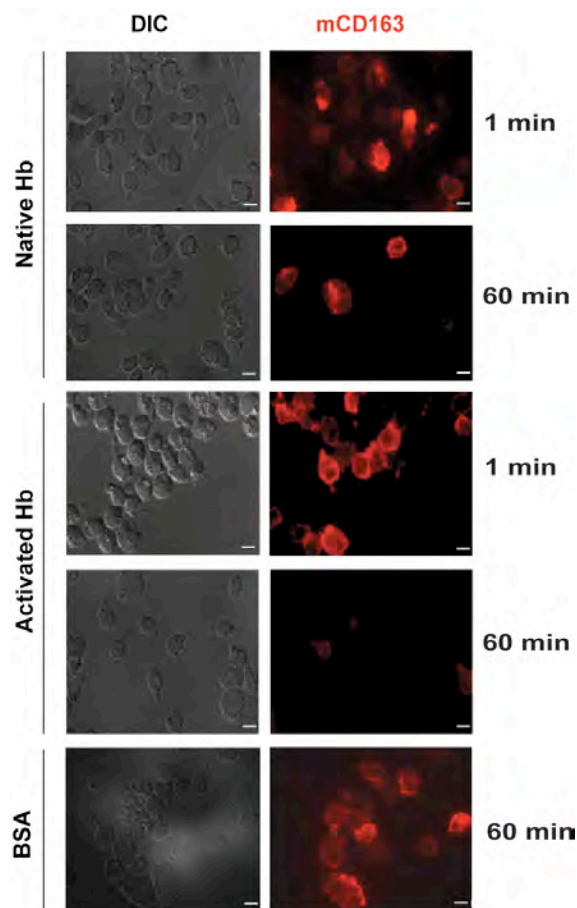


**Figure 3.8: Immunostaining of CD163 on monocytes.** Immunofluorescent staining of CD163 (red) in SU-DHL-1 cells treated with 1 mg/ml of native or activated Hb for up to 60 min. BSA-treated cells served as negative control. Scale bars, 10 μm.

Next, we quantified the amount of sCD163 shed into the culture supernatants of Hb treated monocytes using CD163-specific sandwich ELISA. Reciprocal to mCD163, the level of sCD163 in the culture supernatant increased in a time-dependent manner (**Figure 3.9**). Compared to native Hb, the activated Hb induced twice the amount of shedding by 60 min. Our results suggest that the monocytes shed mCD163 when they encounter Hb, particularly, the redox active Hb-POX.
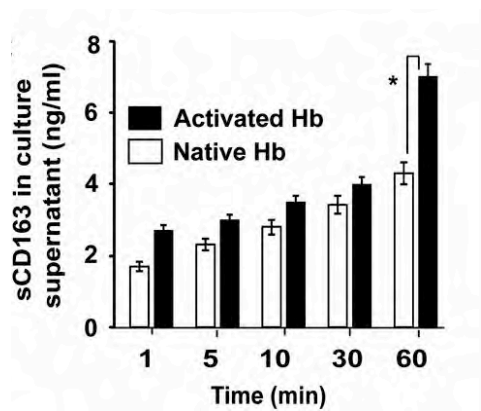


**Figure 3.9: Soluble CD163 in culture supernatants**. sCD163 in the culture supernatants of SU-DHL-1 monocytes treated with 1 mg/ml of native or activated Hb over a time course of up to 60 min was measured using a human CD163-specific sandwich ELISA. * indicates $p < 0.05$.

To preclude any possible effect of endotoxin contamination on the Hb-induced shedding of mCD163, both native and activated Hb were tested and found to contain ≤ 0.05 EU/ml (**Table 3.1**) by the PyroGene assay (refer section 2.2.14). In addition, we measured the CD163 shedding in the presence of polymyxin B (PMB), an endotoxin-neutralizing peptide (David & Sil, 2010). PMB at a concentration of 10 µg/ml has been routinely used in immunological studies to negate the effect of any endotoxin contamination (Brennan et al, 2012; Cardoso et al, 2007). **Figure 3.10** shows that treatment

with 10 µg/ml PMB (Cardoso et al, 2007) for up to 4 h did not affect the Hb-induced shedding of CD163 from monocytes. Consistent with our earlier observations (see Figure 3.7), Hb induced shedding of CD163 within 1 h, which then recovered within 4 h. Cells treated with PMB alone were used as a negative control. This result indicates that the Hb-induced shedding of CD163 is not due to contamination with endotoxin, since PMB would have bound and inactivated the endotoxin.

**Table 3.1:** Endotoxin concentration in native and activated Hb.

| Sample | Endotoxin concentration (EU/ml) |
|---|---|
| Native Hb (1 mg/ml) | 0.04 |
| Activated Hb (1 mg/ml) | 0.05 |
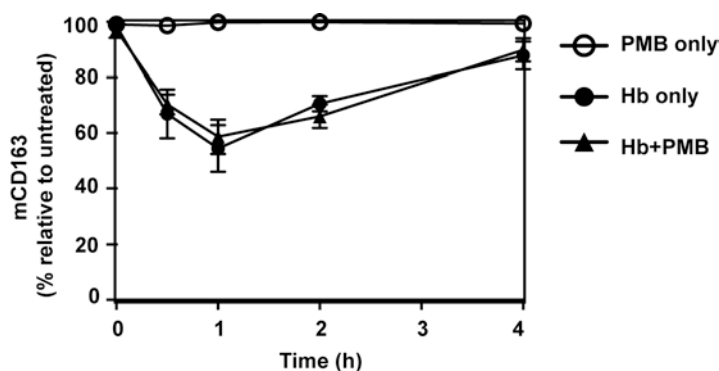


**Figure 3.10: Polymyxin B does not affect Hb-induced CD163 shedding from monocytes.** Flow cytometric analysis of the density of CD163 on SU-DHL-1 cells treated with either 1 mg/ml of Hb alone or in combination with 10 µg/ml of polymyxin B (PMB) over 4 h. Cells treated with PMB alone were used as a negative control.

**3.1.4 sCD163 binds Hb and inhibits Hb-POX activity**

**3.1.4.1 Interaction between sCD163 and Hb**

Since sCD163 has been shown to bind Hb:Hp complex *in vitro* (Moller et al, 2010), we queried whether sCD163 could still bind to Hb when Hp is depleted under conditions of severe hemolysis. We showed that Hb bound directly and dose-dependently to sCD163 in the absence of Hp (**Figure 3.11 A**), with the activated Hb binding more strongly than native Hb. Control protein, BSA did not show any binding to sCD163 implying the specific recognition of Hb by sCD163. Co-immunoprecipitation studies confirmed the specific interaction between sCD163 and Hb (**Figure 3.11 B**). Taken together, the above results suggest that sCD163 might function as an additional Hb scavenger in the plasma under conditions of Hp depletion.
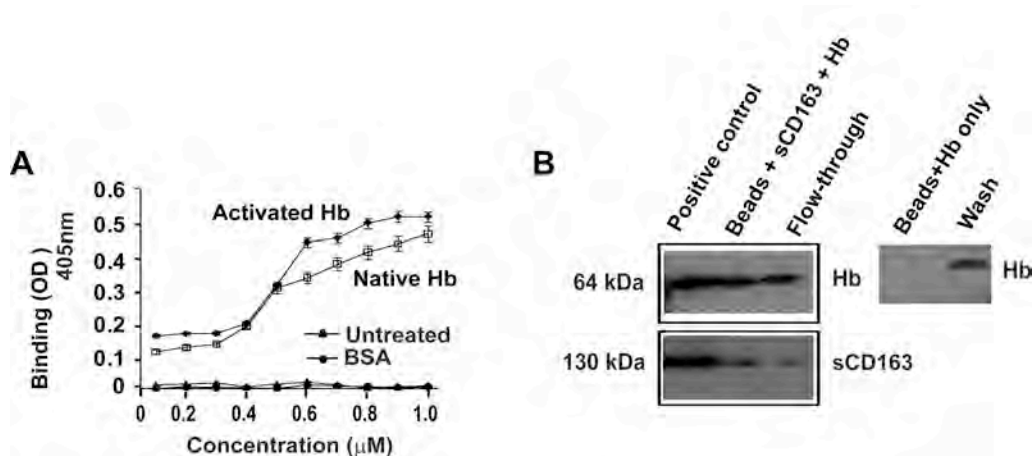


**Figure 3.11: Interaction between sCD163 and Hb.** (**A**) ELISA showing the dose-dependent interaction between sCD163 and 0-1 μM (0-64 μg/ml) native or activated Hb, when 0.1 μg/ml sCD163 was immobilized onto microplates. Untreated refers to the addition of PBS alone. (**B**) Co-immunoprecipitation assay to test for interaction between Hb and sCD163 immobilized on Protein A-conjugated sepharose beads. The purified proteins, sCD163 and Hb were used as positive controls to verify the molecular mass.

### 3.1.4.2 Effect of sCD163 on Hb-POX activity

Redox-active extracellular Hb was reported to aggregate and induce cytotoxicity (Kapralov et al, 2009), hence necessitating the rapid inhibition of Hb-POX even before its uptake into cells. This prompted us to investigate whether binding of sCD163 to Hb could affect the Hb-POX activity extracellularly. We found that the Hb-POX activity decreased significantly and dose-dependently of sCD163, correlating with reaction time (**Figure 3.12**). Within 60 min, up to 70% of the POX activity was suppressed in the presence of 10 ng/ml sCD163, whereas the control protein, BSA, had no effect on the POX activity, confirming the specificity of sCD163 towards Hb. Hence, Figures 3.11 and 3.12 suggest that under conditions of Hp depletion, sCD163 binds and scavenges Hb-POX to protect the host against the damaging ROS.
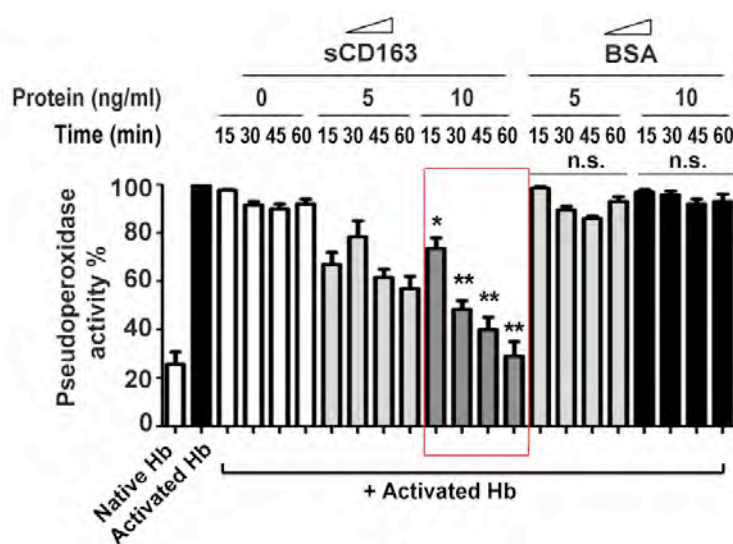


**Figure 3.12: sCD163 dose-dependently attenuates Hb-POX activity.** POX activity of 10 µg activated Hb incubated with sCD163 or BSA (0-10 ng/ml) over a time course of up to 60 min. Red box indicates progressive decrease in Hb-POX activity induced by 10 ng/ml sCD163. * indicates $p< 0.05$; ** $p< 0.005$; n.s. denotes not significant when compared to untreated. Untreated refers to cells treated with PBS.

### 3.1.5 Interaction with IgG facilitates endocytosis of sCD163:Hb complex

### 3.1.5.1 Effect of sCD163 on Hb-induced CD163 shedding and recovery

To query whether sCD163 would influence the level of mCD163 when the monocytes encounter activated cell-free Hb, we supplemented SU-DHL-1 cells with 10 ng/ml purified sCD163 followed by stimulation with 0.1 or 1 μM activated Hb for up to 3 h. The two concentrations of Hb were used because they possessed minimal and maximal binding affinities to sCD163 respectively (Figure 3.11 A). Flow cytometry results indicated that the addition of sCD163 reduced the Hb-triggered shedding of mCD163 (**Figure 3.13 A**). Moreover, the reduction in Hb-induced shedding of mCD163 was also dose-dependent of sCD163 (**Figure 3.13 B**). This suggests that sCD163 exerts a negative feedback on Hb-induced shedding of mCD163, implicating a protective role of sCD163 on mCD163, possibly to maintain the level of mCD163 on the monocytes while sequestering Hb.
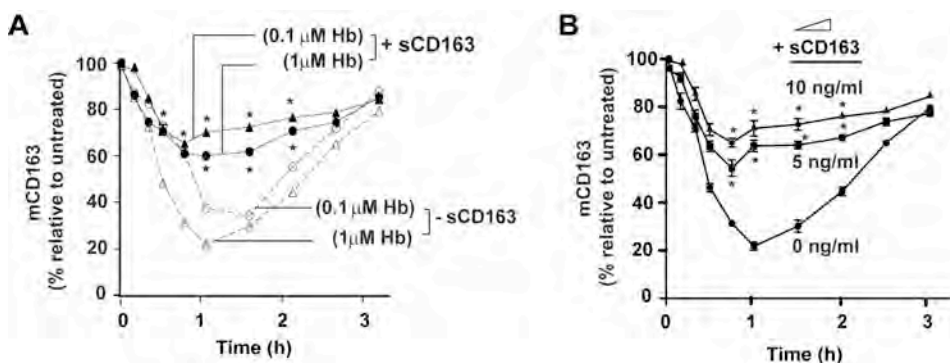


**Figure 3.13: sCD163 promotes mCD163 recovery on monocytes.** (**A**) Flow cytometric analysis showing the residential level of mCD163 in SU-DHL-1 cells treated with 0.1 or 1 μM (6.4 or 64 μg/ml) activated Hb for up to 3 h in the presence or absence of 10 ng/ml sCD163. (**B**) Flow cytometric analysis of mCD163 on SU-DHL-1 cells stimulated with 1 μM activated Hb for up to 3 h in the presence of 0, 5 or 10 ng/ml of sCD163. All the values were normalized against the untreated controls. * indicates $p < 0.05$ when compared with 0 ng/ml sCD163 treated cells.

### 3.1.5.2 Endocytosis of sCD163:Hb complex in the presence of serum

Having shown that sCD163 binds to extracellular Hb and promoted the recovery of mCD163 on the Hb-treated monocytes, we then hypothesized that the sCD163:Hb complex might be recruited back to the monocyte to sequester the Hb-heme iron from the microbial invaders. To test our hypothesis, we first depleted mCD163 on monocytes by phorbol 12-myristate 13-acetate (PMA)-induced shedding and inhibited new synthesis using the protein synthesis inhibitor, cycloheximide (CHX) (**Figure 3.14 A).** Using confocal microscopy, we then tracked the fate of the sCD163:FITC-Hb complex by incubating the complex of sCD163:FITC-Hb (activated form) with the mCD163-deficient monocytes. We found that the complex was recruited to the cell membrane within 15 min (**Figure 3.14 B)** and internalized by 45 min. The control protein, BSA did not bind to the monocytes. Also, the sCD163:Hb complex did not bind to the control HEK293T cells, which are devoid of CD163, implicating the specific recognition of the sCD163:Hb complex by the monocytes. Importantly, sCD163 by itself or the sCD163:FITC-Hb complex, did not bind to monocytes in the absence of serum (**Figure 3.14 C**) suggesting the potential involvement of serum protein(s) in trafficking the sCD163:Hb complex.
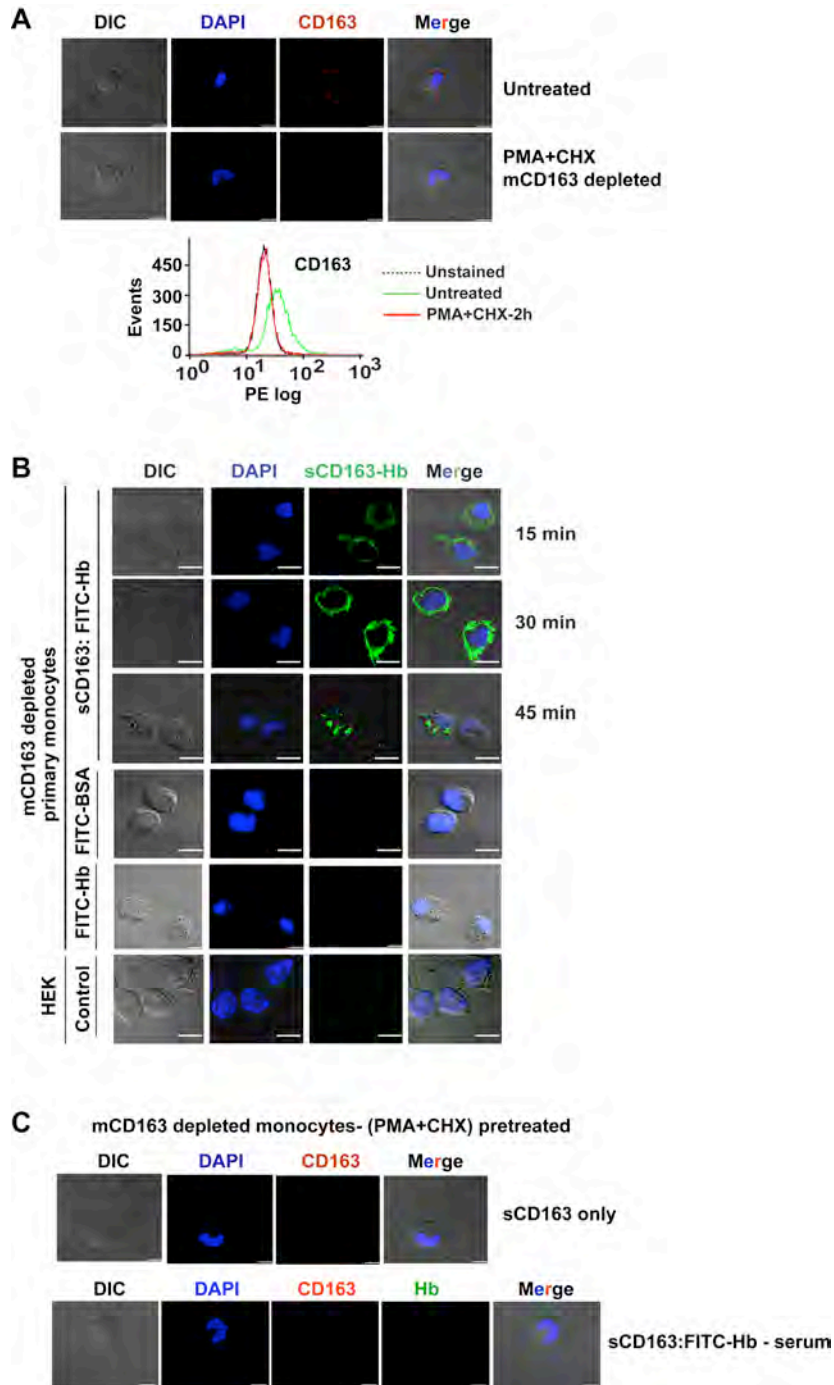
**Figure 3.14: sCD163:Hb complex is endocytosed by monocytes in the presence of serum.** (**A**) Immunostaining (top panel) and FACS analysis (bottom panel) of CD163 in primary monocytes pre-depleted of CD163 by treatment with PMA and CHX for 2h. Untreated refers to cells treated with PBS. Scale bars, 5 μm. (**B**) 0.1 mg/ml of purified sCD163:FITC-activated Hb complex (green) was incubated for 15-45 min with primary monocytes pre-depleted of mCD163 and tracked by confocal microscopy. (**C**) mCD163-depleted primary monocytes were incubated for 30 min with either sCD163 alone or the sCD163:FITC-Hb complex in absence of serum and the cells were immunostained for CD163. Scale bars, 5 μm.

84

To examine whether CD163 from the recruited complex of sCD163: Hb re-appeared as mCD163 or whether the restored level of mCD163 arose from new protein synthesis, we added the protein synthesis inhibitor, CHX (Shankar et al, 2008) to the cells prior to treatment with the sCD163:Hb complex. **Figure 3.15 A and B** shows that CHX treatment did not compromise the recovery of mCD163, indicating that the mCD163 level was not attributable to *de novo* protein synthesis, but rather, it likely originated from the internalized sCD163. Moreover, we found that the complex was recruited to the cell membrane within 15 min and internalized by 45 min. The internalized sCD163 was co-localized intracellularly with transferrin, a marker of early recycling endosomes (Barysch et al, 2009; Hopkins, 1983). By 90 min, the sCD163 re-appeared on the cell membrane, which is consistent with the time at which the Hb-treated monocytes started to recover mCD163 (Figure 3.7). The above results suggest that the sCD163:Hb complex is endocytosed by the monocytes and the endocytosed sCD163 appears to be trafficked to the membrane.
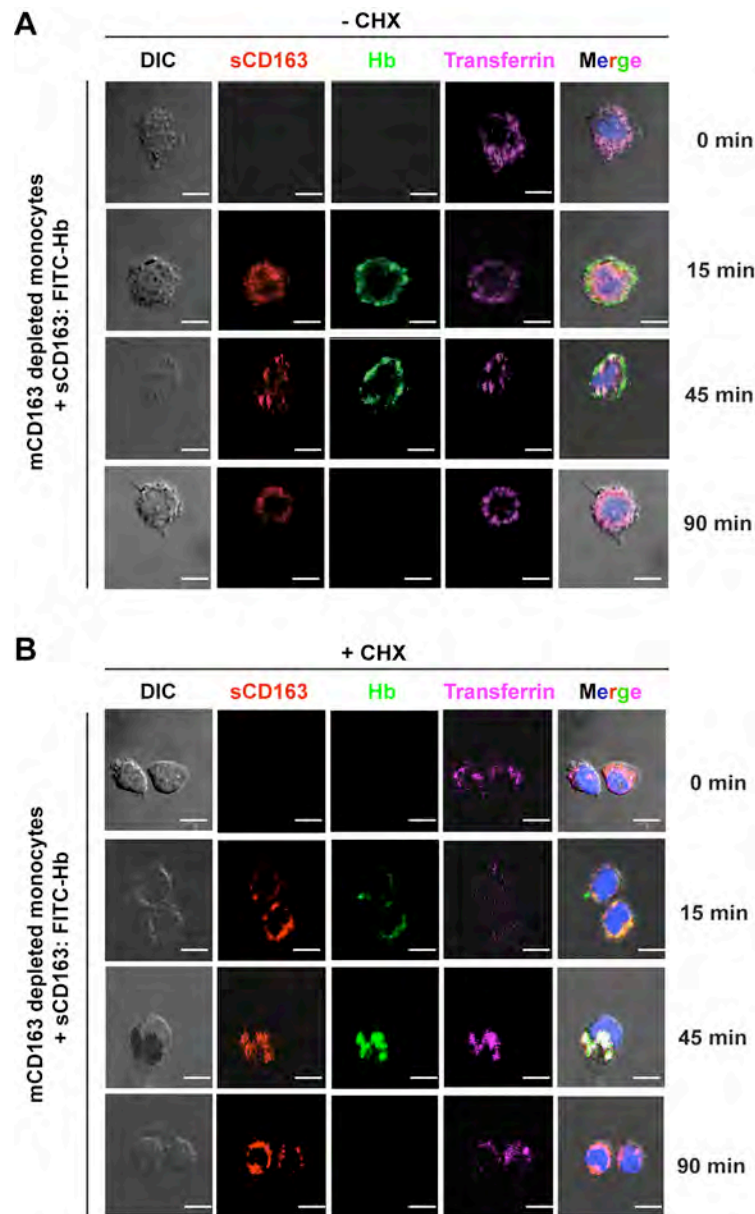
**Figure 3.15: sCD163 from the endocytosed sCD163:Hb complex co-localizes with recycling endosomes.** mCD163 pre-depleted monocytes were incubated with 0.1 mg/ml of sCD163: FITC activated Hb complex and 10 µg/ml of Alexa-647 conjugated transferrin (recycling endosomal marker) over a time course of up to 90 min: (**A**) with or (**B**) without 5 µg/ml cycloheximide (CHX) pre-treatment for 60 min. The localization of sCD163 and Hb was tracked by immunostaining. Images were obtained using the LSM 510 META confocal microscope under 100x oil objective. Scale bars, 5 µm. Images are representative of 3 independent experiments using primary monocytes from a single healthy donor.

**3.1.5.3 Interaction of the sCD163:Hb complex with IgG**

To identify the potential receptor involved in the recruitment of sCD163:Hb complex into monocytes, we tested the possible role of FcγR since it is known to mediate the uptake of oxidized protein complexes from the plasma (Huang et al, 1999). Since FcγR is a known receptor for IgG and IgG-associated immune complexes (Rossman et al, 1989), we examined the potential interaction of IgG with the sCD163:Hb complex. We found that indeed Hb in the sCD163:Hb complex co-immunoprecipitated with IgG from the serum of healthy individuals (**Figure 3.16 A, left panel**). In this experiment, purified human IgG was conjugated to beads followed by incubation with Hb. The Hb:IgG complex was verified using anti-Hb and anti-IgG. Non-specific interaction of Hb with the beads was ruled out by including a control in which Hb was incubated with the beads alone. The sCD163:Hb complex co-immunoprecipitated with IgG conjugated beads (**Figure 3.16 A, right panel**). A negative control was included to show that sCD163 by itself did not interact with IgG. The ELISA results corroborated and established a dose-dependent interaction between sCD163:FITC-Hb complex and IgG immobilized onto the Maxisorp plates (**Figure 3.16 B**). No binding occurred with FITC-BSA control, suggesting that Hb in the sCD163:Hb complex binds to IgG. Furthermore, in the absence of sCD163, Hb displayed reduced affinity for IgG.
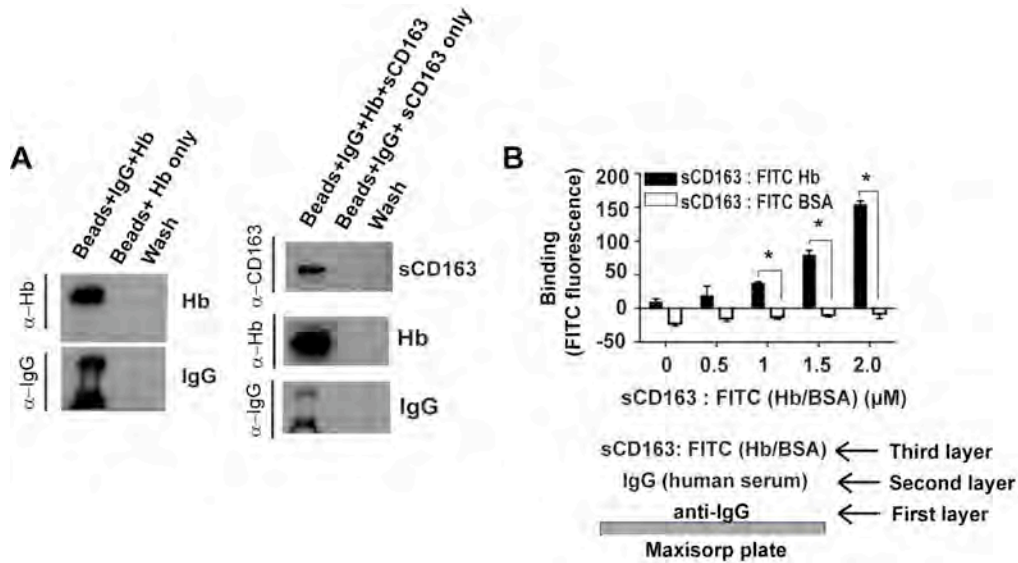
**Figure 3.16: sCD163:Hb complex interacts with IgG.** (**A**) **Left panel**- Co-immunoprecipitation assay to test for interaction between IgG and Hb. Negative control included Hb incubated with beads alone without IgG. **Right panel**- Co-immunoprecipitation assay to test for interaction between IgG, Hb and sCD163. (**B**) ELISA to show the dose-dependent binding of sCD163:FITC-Hb complex to IgG immobilized on Maxisorp plates. FITC-BSA was used as negative control in place of FITC-Hb. All the readings were subtracted from the values obtained with addition of FITC-Hb alone. * indicates *p*< 0.05.

Since sCD163 binds to Hb (Figure 3.11), we sought to test whether purified IgG, Hb and sCD163 would form a complex *in vitro*. Real time biointeraction using surface plasmon resonance analysis showed strong binding between IgG:Hb ($K_D = 1.15x10^{-7}$ M) and between IgG:Hb:sCD163 ($K_D = 2.25x10^{-9}$ M), producing shift and supershift, respectively, in a dose-dependent manner when the proteins were injected successively onto the IgG-immobilized CM-5 chip (**Figure 3.17 A and B**). The specificity of the interaction between Hb and IgG was affirmed by the supershift produced by anti-Hb (**Figure 3.17 C**).
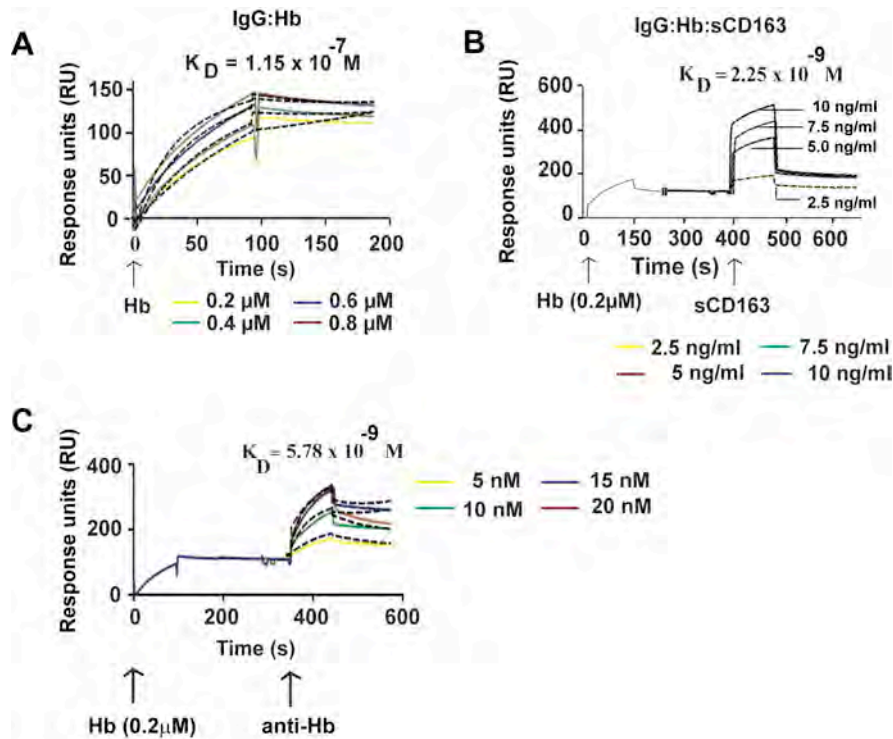
**Figure 3.17: Real-time interaction between Hb, sCD163 and IgG.** (**A-B**) Representative sensograms of 3 independent surface plasmon resonance experiments showing the dose-dependent binding profiles between immobilized IgG to: panel (**A**) 0.2-0.8 μM (12.8-51.2 μg/ml) Hb and panel (**B**) Hb (0.2 μM) + sCD163 (2.5-10 ng/ml). Response units (RU) for panel (A) were dual referenced against BSA-N-acetylglucosamine immobilized reference flow cell and 0.2-0.8 μM (13.3-53.2 μg/ml) BSA, while panel (B) was referenced against sCD163 only (without Hb) controls. Dashed lines represent the curve fitting. (**C**) A representative sensogram of three independent SPR experiments to demonstrate the specificity of interaction between immobilized IgG and Hb. 0.2 μM Hb was injected over IgG immobilized on a CM-5 chip. Then, increasing doses of anti-Hb (5-20 nM) were injected over the bound Hb, where supershifts demonstrated specificity of binding of Hb to IgG. Dashed lines represent the curve fitting.

**3.1.5.4 FcγR facilitates endocytosis of the sCD163:Hb:IgG complex**

To investigate whether the sCD163:Hb:IgG complex was endocytosed via interaction with FcγR on the primary monocytes, we performed flow cytometry after incubation with increasing doses of purified complex of sCD163, FITC-Hb and IgG with wild-type cells and FcγR-knockdown cells.

The efficiency of knockdown of all three iso-types of FcγRs - FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) was verified by the loss of protein at 48 h post-nucleofection (**Figure 3.18 A)**. The FcγR-knockdown cells were stained sequentially using primary antibody against the indicated FcγR iso-type and Alexa-488 conjugated secondary antibody. The signal intensity was measured using the FITC channel of the flow cytometer. The sCD163:Hb:IgG complex was readily endocytosed by wild-type primary monocytes in a dose-dependent manner (**Figure 3.18 B, top panel**). However, the cells knocked down of CD64, CD32 or CD16 showed substantially reduced endocytosis of the sCD163:Hb:IgG complex (**Figure 3.18 B, bottom panel**). CD64 knockdown, in particular, compromised the binding of the sCD163:Hb:IgG complex to the greatest extent when compared to CD32 or CD16 knockdown. This could probably be due to the higher affinity of CD64 towards IgG compared to CD32 or CD16 (Ravetch & Bolland, 2001). Triple knockdown of all the FcγR types almost completely abrogated the binding of the sCD163:Hb:IgG complex to monocytes. The negative controls, BSA (Figure 3.18B, top right panel), sCD163 (Figure 3.14C) and Hb:IgG (Figure 3.18C) did not bind to the cells indicating that sCD163:Hb complex was specifically endocytosed via interaction with IgG, the ligand that bridges the sCD163:Hb complex to FcγR on the monocyte.
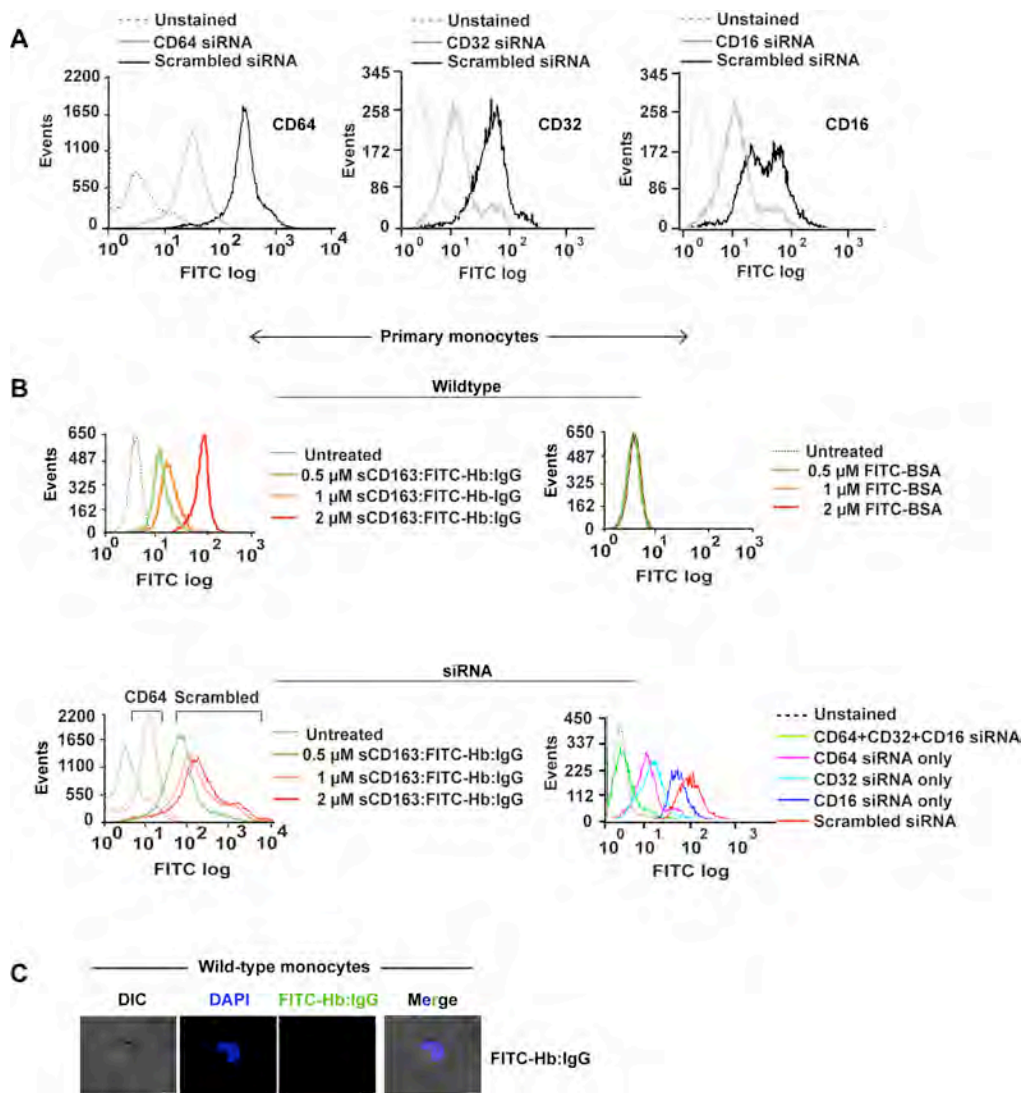
**Figure 3.18: Endocytosis of the sCD163:Hb:IgG complex by FcγR on monocytes.** (**A**) FACS to analyze the efficiency of CD64, CD32 and CD16 knockdown in primary monocytes at 48 h post-nucleofection. Scrambled siRNA-nucleofected primary monocytes were used as negative control. (**B**) Wild-type (**Top panel**), CD64-, CD32- and CD16- knocked-down primary monocytes (**Bottom panel**) were incubated with 0.5-2 μM (32-128 μg/ml) sCD163:FITC-Hb:IgG complex for 30 min and endocytosis was quantitated using the CyAn ADP flow cytometer on the FITC channel. FITC-BSA was used as a negative control. Untreated denotes cells treated with PBS only. (**C**) Confocal microscopy to test the binding of the negative control FITC-Hb:IgG to wild-type monocytes. Scale bars, 5 μm. Data are representative of 3 independent experiments using primary monocytes from a single healthy donor.

91

### 3.1.6 Fate of endocytosed CD163 and Hb

### 3.1.6.1 Endocytosed sCD163 is recycled to membrane

Next, we quantified and tracked the subcellullar localization of CD163 after endocytosis of sCD163:Hb:IgG complex into primary monocytes. The monocytes were pre-depleted of mCD163 followed by treatment with CHX to block subsequent *de novo* synthesis of CD163. Results showed that within 15 min, sCD163 was detected in the membrane fraction, indicative of binding of the sCD163:Hb:IgG complex to the membrane (**Figure 3.19**). Within 30-45 min, sCD163 was localized in the cytoplasm, corroborating the endocytosis of the sCD163:Hb:IgG complex, and this was effectively blocked by pre-treatment with chlorpromazine, an inhibitor of endocytosis (Mueller et al, 2002). By 90 min, the internalized CD163 re-appeared on the membrane and this was abolished when the cells were pre-treated with monensin, a known inhibitor of recycling endosomes (Schaer et al, 2007). When the cells were simultaneously pre-treated with both chlorpromazine and monensin, CD163 was only observed on the cell membrane throughout the 90-min duration, indicating that both the endocytosis of the sCD163:Hb:IgG complex and subsequent recycling of the endocytosed CD163 were compromised. This result is in agreement with our earlier observations showing co-localization of the endocytosed sCD163 with recycling endosomes by 45 min and reappearance of sCD163 on the cell membrane by 90 min (Figure 3.15).
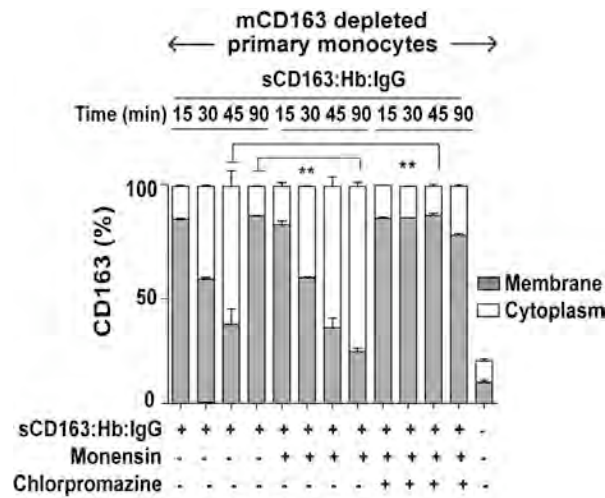
**Figure 3.19: Inhibitors of endocytosis and recycling inhibit subcellular trafficking of endocytosed sCD163.** Primary monocytes pre-depleted of mCD163 and treated with 5 µg/ml cycloheximide, were incubated with 0.1 mg/ml of the sCD163:Hb:IgG complex over a time course of up to 90 min with or without pretreatment with 70 µM chlorpromazine (inhibitor of endocytosis) and 20 µM monensin (inhibitor of early endosome recycling) for 60 min. CD163 was quantified in the membrane and cytosol fractions, respectively, using sandwich ELISA. ** represents $p<0.005$.

To validate the recycling of sCD163 into mCD163 after the uptake of sCD163:Hb:IgG complex, we incubated primary monocytes with either biotinylated-sCD163 alone or as a preformed complex of biotin-sCD163:Hb:IgG and tracked the subcellular localization of sCD163 using streptavidin conjugated to HRP. The purity of the membrane/cytosol fractions was assessed using membrane (CD64) or cytosolic (tubulin) markers (**Figure 3.20, top panel**). By 15 min, sCD163 was detected in the membrane fraction and it was endocytosed within 30-45 min (**Figure 3.20, bottom panel**). By 90 min, sCD163 re-appeared on the membrane, consistent with Figure 3.19, validating that sCD163 from the endocytosed sCD163:Hb:IgG complex was

recycled to the membrane. Control cells treated with PBS or sCD163 alone or the sCD163:Hb complex did not show endocytosis and recycling of sCD163, suggesting the specific uptake of the sCD163:Hb:IgG complex by monocytes. Thus far, the results corroborate that mCD163 plays a major role in frontline defense as it binds Hb to reduce the POX activity, while the shedded sCD163 further scavenges plasma Hb, re-enters the monocyte and undergoes recycling into mCD163, thus completing the autocrine cycle of Hb detoxification and CD163 renewal.
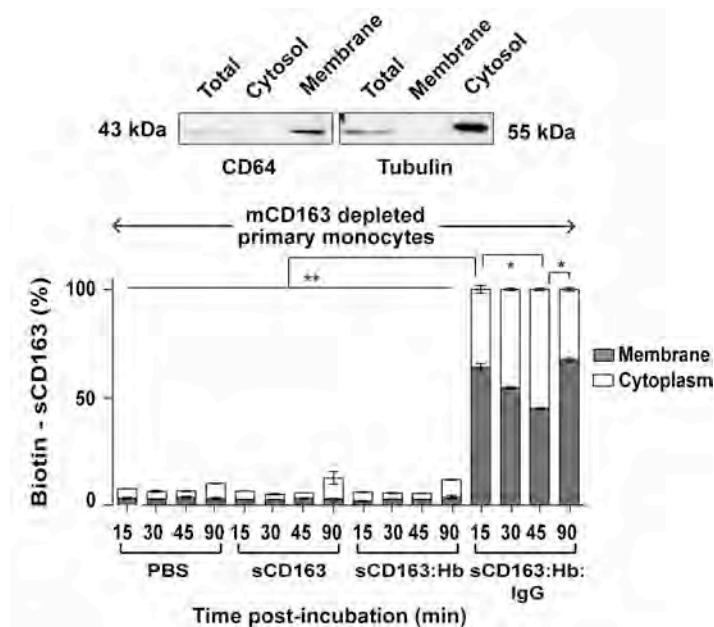


**Figure 3.20: Endocytosed sCD163 is recycled into mCD163 on monocytes. Top panel**- Purity of membrane/cytosol fractions was tested using membrane (CD64) or cytosolic (tubulin) markers. **Bottom panel**- mCD163 depleted primary monocytes were incubated with either biotinylated sCD163 alone or biotin-sCD163:Hb:IgG complex over a time course of up to 90 min. Biotinylated sCD163 was quantified in membrane or cytosolic fractions using streptavidin-HRP by ELISA. * represents $p < 0.05$; ** represents $p < 0.005$.

### 3.1.6.2 Bioinformatics prediction of post-translational modifications of sCD163

Next, we considered the potential mechanism by which the endocytosed sCD163 was recycled to the membrane to restore mCD163 on the monocytes. Towards this goal, we used bioinformatics software(s) to predict the potential sites at which sCD163 might be post-translationally modified in order to gain membrane anchorage. The four major post-translational modifications, involving covalent attachment of lipids to proteins are:

1. Myristoylation- addition of myristate (C14 saturated acid)

2. Palmitoylation- addition of palmitate (C16 saturated acid)

3. Prenylation- addition of isoprenoid group (e.g. farnesol, geranylgeraniol)

4. Glycosylphosphatidylinositol (GPI) anchor

sCD163 is a homogenous protein released upon proteolytic cleavage in the SRCR domain 9 of the mCD163. The sCD163 spans ~94% of the extracellular domain of mCD163 (**Figure 3.21**). Mass spectrometry revealed that the most N-terminal peptide sequence of sCD163 corresponds to amino acids (aa) 48-58 of mCD163 in domain 1, while the most C-terminal peptide is located in domain 9 and corresponds to amino acids 973-998 of mCD163 (Moller et al, 2010). Results from the computational predictions indicated that there were no potential myristoylation, prenylation or GPI anchorage sites in sCD163. However, there were potential palmitoylation sites near the C-terminal region of sCD163 (**Table 3.2**).

95

Palmitoylation is known to enhance the surface hydrophobicity and membrane affinity of protein substrates, and play important roles in modulating protein trafficking (Draper et al, 2007; Linder & Deschenes, 2007), stability (Linder & Deschenes, 2007), and sorting (Greaves & Chamberlain, 2007).
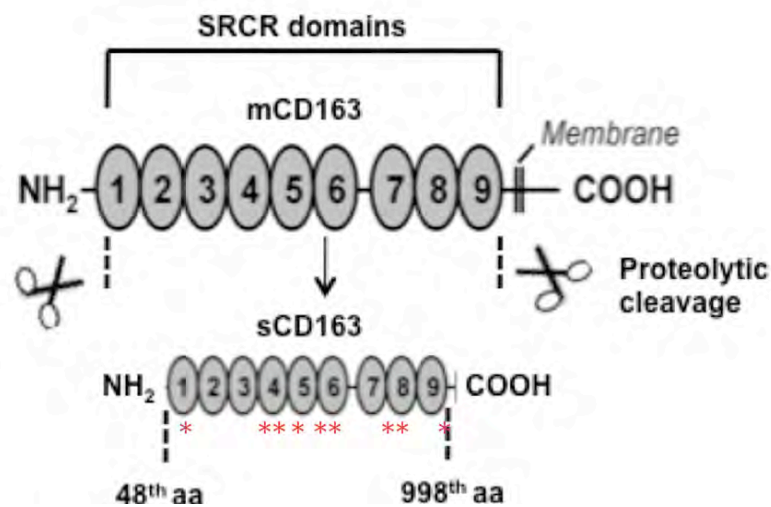


**Figure 3.21: Proteolytic cleavage of mCD163 into sCD163.** sCD163, which is produced as a result of proteolytic cleavage of mCD163 by matrix metalloproteinases, comprises nearly 94% of the extracellular scavenger receptor cysteine rich (SRCR) domains of mCD163. The most N-terminal peptide corresponds to amino acids (aa) 48-58 of mCD163 while the most C-terminal peptide corresponds to amino acids 973-998. * indicates the distribution of the predicted palmitoylation sites in sCD163.

CSS-Palm 3.0 (Zhou et al, 2006) was used to predict the potential palmitoylation sites in the sCD163 under the medium threshold filter. The C-terminal most palmitoylation site is Cys998, which is mapped to the extracellular domain 9 of mCD163, corresponding to the region at which mCD163 is proteolytically cleaved to release the soluble form (refer Figure 3.21) (Moller et al, 2010).

**Table 3.2: Predicted palmitoylation sites in sCD163.**

| Position from N-terminus | Peptide | Score | Cutoff | Cluster |
|---|---|---|---|---|
| 94 | VICNQLGCPTAIKAP | 0.205 | 0.196 | Cluster A |
| 398 | QRLLGKVCDRGWGLK | 0.2 | 0.196 | Cluster A |
| 442 | TWLFLSSCNGNETSL | 0.281 | 0.196 | Cluster A |
| 487 | LVGGDIPCSGRVEVK | 0.205 | 0.196 | Cluster A |
| 672 | TALGASLCPSEQVAS | 0.248 | 0.196 | Cluster A |
| 682 | EQVASVICSGNQSQT | 0.248 | 0.196 | Cluster A |
| 864 | ETTVGVVCRQLGCAD | 0.357 | 0.196 | Cluster A |
| 869 | VVCRQLGCADKGKIN | 0.267 | 0.196 | Cluster A |
| 998 | IWLNEVKCKGNESSL | 0.248 | 0.196 | Cluster A |

CSS-Palm 3.0 was used to predict the potential palmitoylation sites in sCD163 under the medium threshold filter. Yellow highlight shows that the C-terminal most palmitoylation site is Cys 998 (red) and this corresponds to the cleavage site from mCD163.

### 3.1.6.3 Internalized sCD163 is palmitoylated

Following from the computational analysis, which indicated that the sCD163 could be potentially palmitoylated at the C-terminus to enable it to anchor to the cell membrane upon recycling, experiments were performed to validate the palmitoylation of sCD163. We performed the biotin switch assay (refer section 2.2.9) in mCD163-depleted primary monocytes, treated with the sCD163:Hb:IgG complex for up to 4 h with or without pre-treatment with inhibitors of palmitoylation or myristoylation.

**Figure 3.22** shows that only when treated with the sCD163:Hb:IgG complex, did the palmitoylated CD163 exchange biotin in the presence of the cleavage agent, hydroxylamine (HA) and hence was effectively pulled down by neutravidin beads. This was abolished when the cells were pre-treated with the palmitoylation inhibitor, 2-bromo palmitate (2-BP) but not control myristoylation inhibitor, hydroxymyristic acid (2-HM). In addition, cells pre-treated with chlorpromazine and monensin (inhibitors of endocytosis and recycling respectively) did not show any band corresponding to the palmitoylated CD163. Moreover, palmitoylated CD163 was not pulled down from control cells treated with either sCD163 alone or sCD163:Hb alone, suggesting that sCD163 from the endocytosed sCD163:Hb:IgG complex is palmitoylated and recycled to the membrane.
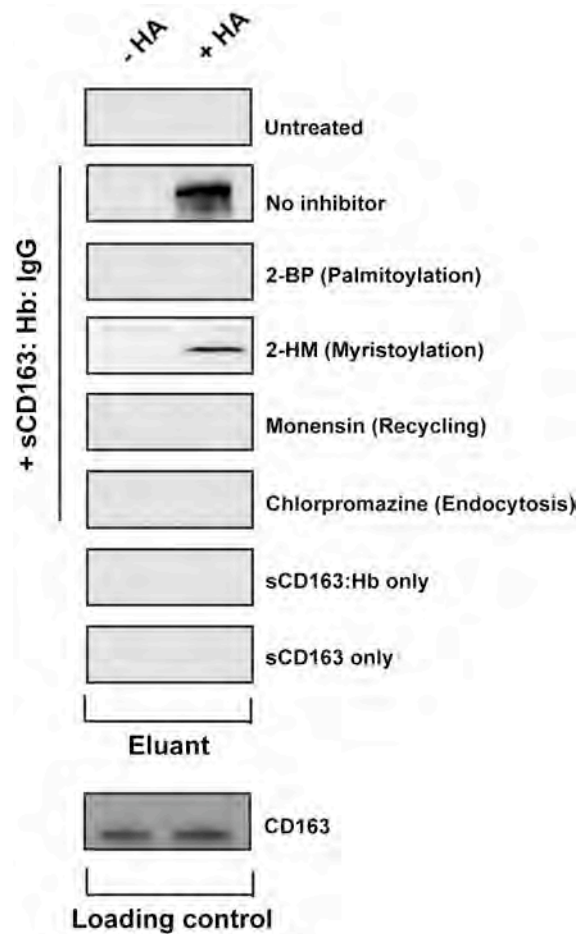
**Figure 3.22: sCD163 from the endocytosed sCD163:Hb:IgG complex is palmitoylated and recycled to the membrane.** Cell-lysates from primary monocytes treated with sCD163 alone or sCD163:Hb or sCD163:Hb:IgG complex with or without pre-treatment with the indicated inhibitors were subjected to the biotin switch assay using the S-acyl group cleavage reagent, hydroxylamine (HA). Proteins that were palmitoylated and hence incorporated biotin were pulled down using neutravidin beads and blots were probed for CD163. Untreated refers to cells treated with PBS only. Loading control shows that equal amounts of total CD163 in the cell-lysate were loaded on to the neutravidin beads.

99

**3.1.6.4 Endocytosed Hb activates heme-oxygenase-1 (HO-1)**

While the endocytosed sCD163 is palmitoylated and recycled to the cell surface via recycling endosomes, we next queried the fate of the internalized Hb. Heme-oxygenase-1 (HO-1) is an enzyme responsible for the catabolism of the redox-active heme into biliverdin, carbon monoxide and iron (Grochot-Przeczek et al, 2012). Results showed that stimulation of monocytes with 1 µM (64 µg/ml) of native Hb induced a 70% increase in the HO-1 activity relative to the control protein, BSA (**Figure 3.23**). Importantly, 1 µM (64 µg/ml) activated Hb induced 20% higher HO-1 activity compared to native Hb. In addition, HO-1 induction by native and activated Hb was dose-dependent. Cells treated with BSA did not show any activation of HO-1, implying the specific activation of HO-1 by endocytosed Hb. Conceivably, this timely induction of HO-1 activity catabolizes heme from the internalized Hb into biliverdin, which is further converted into harmless bilirubin by biliverdin reductase.
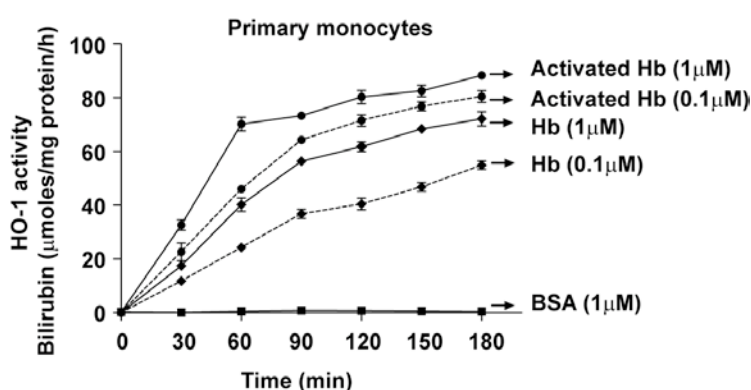


**Figure 3.23: Endocytosed Hb activates HO-1 in monocytes.** Primary monocytes were stimulated with 0.1, 1 µM (6.4, 64 µg/ml) of native or activated Hb over a time course of up to 180 min and the HO-1 activity (µmoles bilirubin/mg protein/h) was measured by spectrometric quantitation of bilirubin in the presence of excess substrate. Cells treated with BSA served as negative control.

**3.1.7 Hb induces monocyte-endothelial crosstalk via sCD163 and IgG**

**3.1.7.1 sCD163:Hb:IgG complex upregulates HO-1 in endothelial cells**

Next, we queried the cellular physiological significance of the monocyte-derived sCD163. Since monocytes are in contact with the vascular endothelial cells *in vivo*, it is conceivable that the endothelial cells would encounter sCD163 during hemolysis. The sCD163 could potentially act in a paracrine fashion to communicate and alert the proximal cells of the imminent presence of cytotoxic Hb. To test this hypothesis, we used primary human dermal microvascular endothelial cells (HMVEC), which are known to endogenously express FcγRII (CD32), (Groger et al, 1996) but lack CD163 (Hiraoka et al, 2005). We then measured the induction of HO-1 in HMVEC cells incubated for 6 h with increasing doses of the sCD163:Hb:IgG complex. Results showed that when compared to just Hb alone or other negative controls, the sCD163:Hb:IgG complex upregulated HO-1 levels by 3-fold (**Figure 3.24**). The induction of HO-1 was dose-dependent of the sCD163:Hb:IgG complex (Figure 3.24*,* box). Cells treated with hemin, which is the substrate of HO-1, were used as positive control. This indicates that sCD163 and IgG mediate the Hb-induced transactivation of the endothelial cells. After internalization into HMVEC cells, Hb upregulates the heme catabolizing enzyme, HO-1.
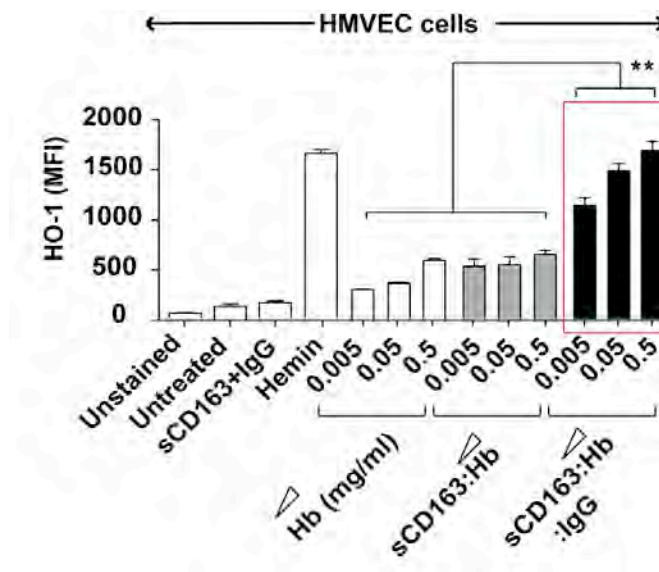
**Figure 3.24: HO-1 upregulation by sCD163:Hb:IgG complex in endothelial cells.** HMVEC cells were stimulated with increasing doses of Hb alone or pre-formed sCD163:Hb:IgG complex for 6 h and flow cytometry was used to quantify the protein level of HO-1. Hemin-treated cells were used as positive control. Untreated refers to cells treated with PBS alone. \*\*indicates *p*< 0.005.

**3.1.7.2 Synergistic cytokine production in CD163^{+} monocyte-endothelial co-culture**

To assess the potential Hb-induced crosstalk between the monocytes and endothelial cells, we co-cultured the two cell-types in the presence of Hb, and measured the cytokine production by the cells. To confirm the significance of CD163 in this process, we employed CD163^{+} primary monocytes or CD163^{-} THP-1 monocytes (control) (Bachli et al, 2006). **Figure 3.25 A** (box) shows a significant increase in the production of TNF-α, IL-8 and IL-10, when the HMVEC cells were co-cultured with CD163^{+} primary monocytes compared to THP-1 or when stimulated in isolation. This synergy was lost when the monocytes were pre-incubated with anti-CD163, suggesting that the

monocyte-derived sCD163 is indispensable for the activation of endothelial cells, which lacks endogenous CD163. Furthermore, stimulation of HMVEC with the sCD163:Hb:IgG complex elicited higher amounts of TNF-$\alpha$ and IL-8 compared to cells treated with the individual protein controls, (**Figure 3.25 B)** corroborating our co-culture results (Figure 3.25 A). In particular, we observed higher IL-10 production by Hb-treated monocyte-HMVEC co-culture when compared to isolated HMVEC cells likely due to the activation of HO-1, which has been implicated in mediating IL-10 production (Drechsler et al, 2006). Thus, the monocyte-derived sCD163 mediates the paracrine activation of the proximal endothelial cells to systemically alert the human body on the imminent toxicity of plasma Hb.
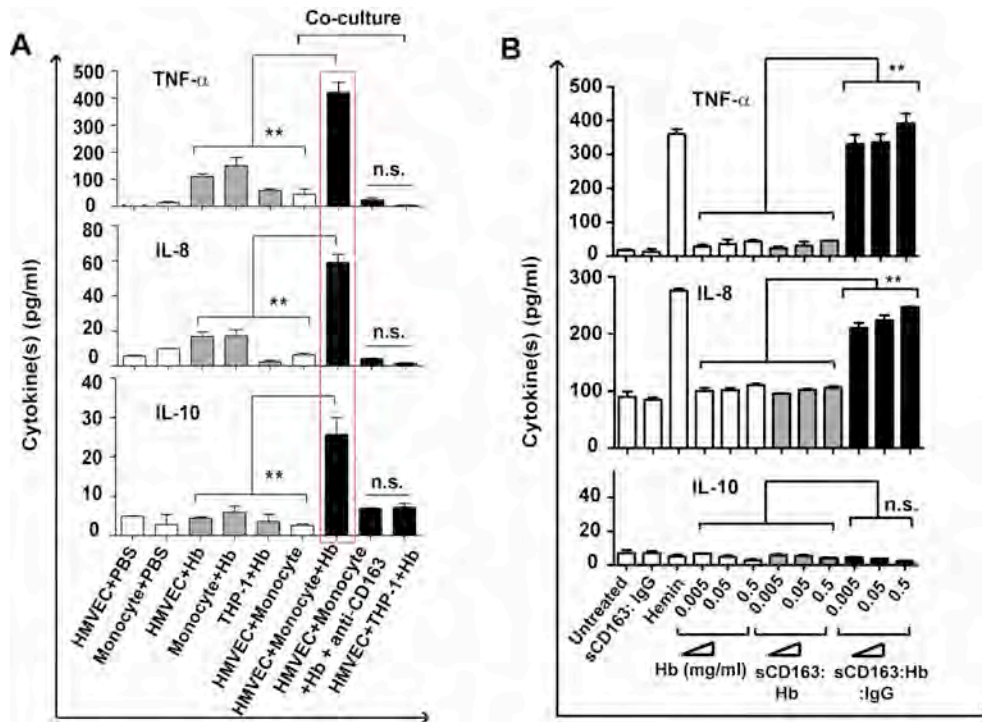
**Figure 3.25: Hb-induced synergistic cytokine production in endothelial cells co-cultured with CD163$^+$ monocytes.** (**A**) Cytokine production when HMVEC cells were co-cultured for 24 h with CD163$^+$ primary monocytes or CD163$^-$ THP-1 cells in the presence of 0.5 mg/ml Hb. (**B**) Cytokine production by HMVEC cells when stimulated for 24 h with increasing doses of Hb alone or pre-formed sCD163:Hb:IgG complex. Untreated refers to cells treated with PBS alone. ** indicates $p < 0.005$; n.s. denotes not significant.

### 3.1.7.3 Tracking CD163 and Hb in CD163$^+$ monocyte-endothelial co-culture

Next, we tracked CD163 and Hb in the HMVEC, which had been co-cultured with primary monocytes or THP-1 monocytes for 45 min. **Figure 3.26** shows that both CD163 and Hb are co-localized in HMVEC only when co-cultured with CD163$^+$ primary monocytes but not with CD163$^-$ THP-1 cells. Consistently, the co-localization of Hb and CD163 within the HMVEC was observed only when the two proteins were presented as a complex of sCD163:Hb:IgG (**Figure 3.27**). Importantly, in the absence of IgG, no

endocytosis of Hb was detected in HMVEC, even if co-cultured with CD163^{+} monocytes, suggesting that IgG is required to bridge the sCD163:Hb complex to FcγR on the HMVEC. In addition, when HMVEC and CD163^{+} monocytes were co-cultured in the absence of Hb, no CD163 entered the HMVEC. By live-cell imaging, we have demonstrated the sCD163-mediated interaction between monocytes and the proximal endothelial cells in the presence of Hb and IgG (**Videos 8-11**).
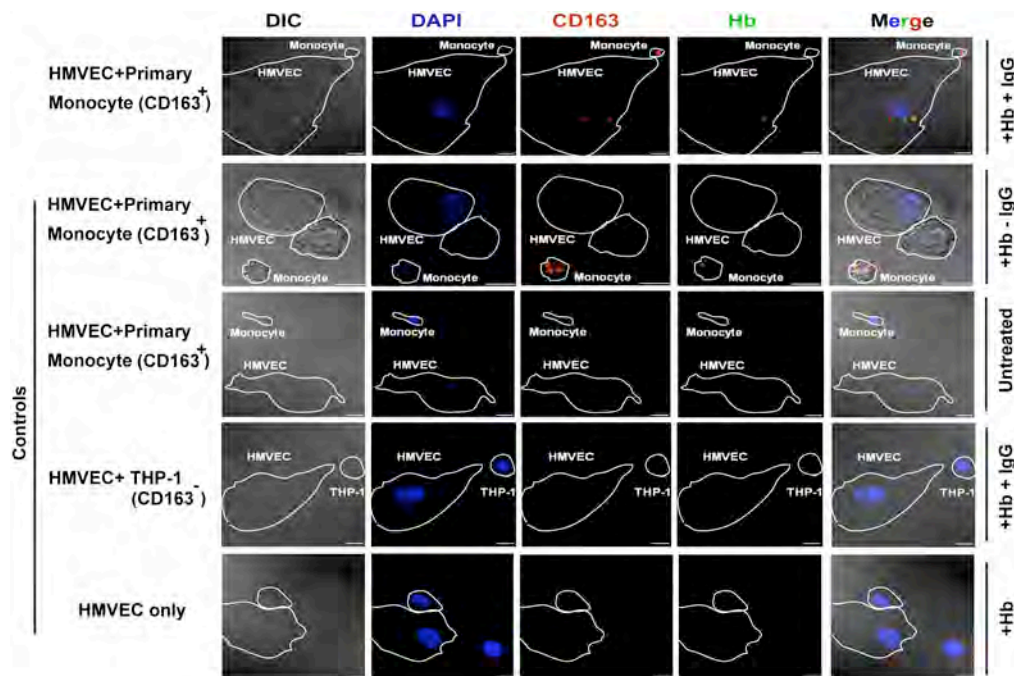


**Figure 3.26: Co-localization of sCD163 and Hb in endothelial cells co-cultured with CD163^{+} monocytes.** Immunostaining to track localization of sCD163 and Hb in HMVEC cells co-cultured with either CD163^{+} primary monocytes or CD163^{-} THP-1 cells in the presence of Hb for 45 min. Untreated refers to cells treated with PBS. The cell-boundary is marked in white. Images were obtained using the LSM 510 META confocal microscope under 100x oil objective. Scale bars, 10 μm.
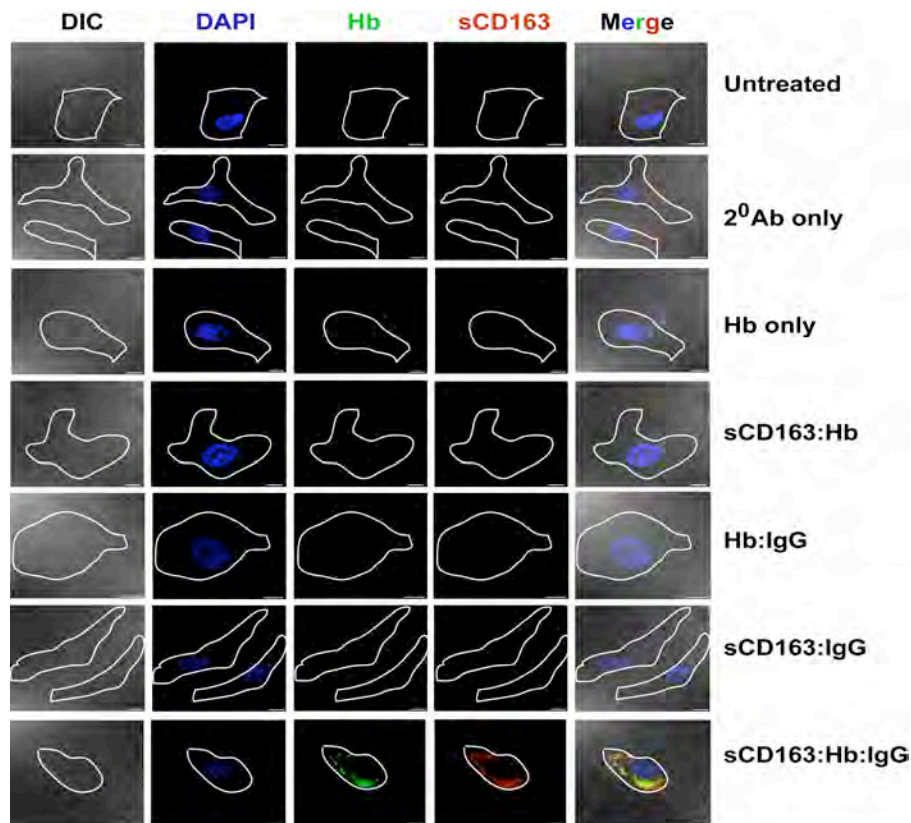
**Figure 3.27: Co-localization of sCD163 and Hb in endothelial cells stimulated with sCD163:Hb:IgG complex.** Immunostaining to track sCD163 and Hb in HMVEC cells incubated with pre-formed sCD163:Hb:IgG complex for 30 min. Untreated refers to cells treated with PBS. The cell-boundary is marked in white. Images were acquired using LSM510 confocal microscope under the 100x oil objective. Scale bars, 10 μm.

Taken together, in **Section 3.1** we have discovered and mapped in detail, a novel two-pass mechanism of Hb-detoxification by CD163, independent of Hp, which is the primary anti-oxidant of Hb in plasma. Such an alternative pathway could become operative when Hp is exhausted during severe hemolytic conditions. Firstly, at the outset of the encounter with plasma Hb, mCD163 directly inhibits the Hb-POX activity *in-situ* by binding both the native and infection-activated Hb. Consequently, CD163 also rescues monocytes from Hb-induced apoptosis.

Besides suppressing the Hb-POX activity at the monocyte membrane, CD163 which is co-translocated into the cells, also downregulates the generation of intracellular ROS from the endocytosed Hb. In the absence of such a mechanism, as demonstrated here with CD163$^-$ cells, the hydrophobic nature of the Hb-heme (Vercellotti et al, 1994) could readily permeate the cells, inducing free radicals, which would lead to lipid peroxidation and cell death. Upon endocytosis, the Hb-heme is quickly degraded by HO-1 into biliverdin, carbon monoxide and iron, which is incorporated into iron-chaperone proteins like transferrin.

Having established the direct inhibition of the redox activity of Hb by mCD163, independent of Hp, we then queried the pathophysiological significance of sCD163 under severe hemolytic condition. We found that sCD163 binds excess plasma Hb dose-dependently and rapidly downregulates the Hb-POX activity. Thus, it is conceivable that during a severe hemolytic episode, such a "capture and quench" action by sCD163 would constitute an effective host defense strategy to sequester the heme iron and pre-empt its redox activity. Of particular importance is that the resulting sCD163:Hb

complex, which is still redox-active, must be rapidly and efficiently removed from circulation so as to subvert the Hb-iron mediated cytotoxicity. To this end, we identified IgG as a novel interaction partner participating with the sCD163:Hb complex to enable endocytosis of the sCD163:Hb:IgG complex via FcγR into the monocytes.

Following endocytosis of the sCD163:Hb:IgG complex, the internalized sCD163 undergoes palmitoylation and is recycled via early endosomes to the cell membrane to restore mCD163. During severe hemolysis, such a dynamic and efficient recycling of sCD163 would presumably potentiate the recovery of mCD163, which acts to fortify the monocytes against the cytotoxic avalanche of free radicals generated by the cell-free Hb-POX. Furthermore, using co-culture experiments, we established that sCD163 in collaboration with IgG, confers Hb-scavenging ability to the proximal endothelial cells (CD163⁻) and also transactivates them to respond against the Hb. Such a crosstalk between monocytes and endothelial cells, which is mediated by the sCD163:Hb:IgG complex via FcγR mounts a systemic and stronger defense against the toxic Hb.

Overall, CD163 is dynamically deployed in a two-pass detoxification tactic to engage with and suppress the pro-oxidative activity of plasma Hb, while its residential level on the monocyte membrane is restored to homeostasis in an efficient <u>autocrine</u> cycle. Simultaneously, it also transactivates adjacent endothelial cells in a <u>paracrine</u> fashion to metabolize the endocytosed Hb, and secrete cytokines to systemically alert the imminent presence of a danger molecule, Hb (**Figure 3.28**).
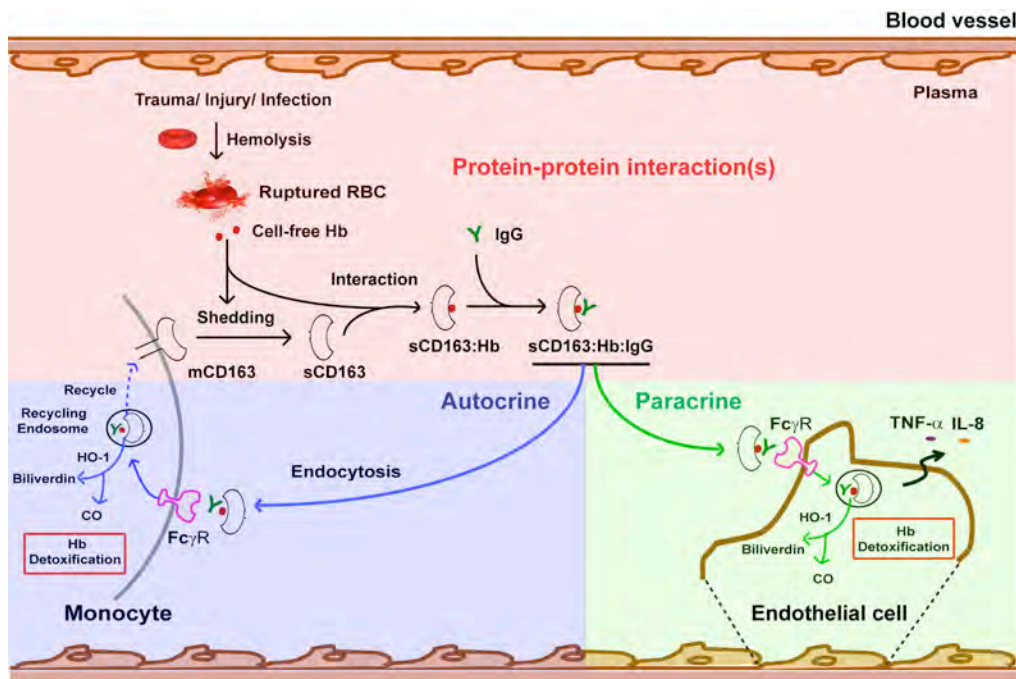
**Figure 3.28: A hypothetical model of Hp-independent intravascular detoxification and clearance of cell-free Hb by CD163.** Hemolysis ruptures red blood cells and releases cytotoxic Hb into the plasma. Upon recruiting Hb, the mCD163 directly suppresses the pseudoperoxidase activity of Hb *in-situ* on the monocyte membrane. Hb induces shedding of mCD163 into the plasma and the resulting sCD163 further "captures and quenches" the residual redox-reactive Hb. Subsequently, IgG interacts with the sCD163:Hb complex and the sCD163:Hb:IgG complex- (i) elicits an autocrine loop of endocytosis via FcγR on the monocyte and subsequent recycling of the internalized sCD163 via endosomes to restore mCD163 homeostasis, while the internalized Hb is catabolized by HO-1 and (ii) induces the paracrine transactivation of the neighboring endothelial cells (represented by HMVEC cells tested in this study) lining the blood vessel causing them to upregulate HO-1 and secrete cytokines to mount a systemic defense against Hb.

## 3.2 Hb-loaded macrophages constitute a silent survival niche for intracellular pathogens

Since cell-free Hb is efficiently endocytosed by the macrophages during hemolytic episodes, we next wanted to investigate the effect of the Hb-priming on the host cell viability and intracellular bacterial clearance during a hemolytic infection.

Plasma Hb plays dual roles in innate immunity; the heme-iron is a nutrient source for pathogens, (Pishchany & Skaar, 2012) and its inherent pseudoperoxidase (POX) activity, which generates ROS, elicits anti-microbial defense (Du et al, 2010; Jiang et al, 2007). To clear the pro-oxidative Hb from circulation, the phagocytes would endeavor to uptake and hence become effectively loaded with Hb. Unfortunately, under these circumstances, infection would conceivably allow opportunistic intracellular pathogens and the endocytosed Hb to be in functional contact with each other. Opportunistic pathogens such as *Staphylococcus aureus* are known to utilize the heme-iron for survival (Skaar et al, 2004). Consistently, patients with hemolytic disorders such as malaria and paroxysmal nocturnal hemoglobinuria (Olsson et al, 2012) are predisposed to infections (Berkley et al, 2009; Scott et al, 2011) especially by intracellular bacteria (Berkley et al, 2009; Bronzan et al, 2007; Mabey et al, 1987). Other studies in mice have also shown that hemolysis increases susceptibility to bacterial infections (Kaye & Hook, 1963a; Kaye & Hook, 1963b; Kaye et al, 1965). Hence, although there is evidence to suggest that hemolysis renders susceptibility to infections, the underlying molecular mechanism remains unclear. This prompted us to hypothesize that Hb,

liberated during hemolysis, promotes susceptibility of phagocytes to intracellular infections.

We used *Staphylococcus aureus* and *Salmonella typhimurium* as representative intracellular bacteria, because they have been reported to survive intracellularly in phagocytes during infection (Clement et al, 2005; Ibarra & Steele-Mortimer, 2009). As a control, we used *Pseudomonas aeruginosa*, which has been reported to reside extracellularly during infection (Yuan et al, 2012). We examined the effect of Hb-priming on the intracellular bacterial clearance by CD163$^+$ macrophages during infection by *Staphylococcus aureus* or *Salmonella enterica serovar typhimurium*. Next, we examined the Hb-POX mediated ROS production and downstream signaling pathways during infection in Hb-primed macrophages. Furthermore, the consequence of infection in Hb-primed macrophages on apoptosis, cytokine production and infectivity of neighboring cells was also investigated.

### 3.2.1 Hb priming enhances survival of intracellular bacteria in macrophages

### 3.2.1.1 Rationale for using U937-derived macrophages

U937-derived macrophages have been widely used as a reliable model system to study the survival and replication of intracellular bacteria (Gao et al, 1998). U937-derived macrophages, which endogenously express FcγR(Looney et al, 1986) and hence efficiently phagocytosed bacteria, were used rather than SU-DHL-1 cells that lack FcγR (Epstein et al, 1978) for bacterial phagocytosis. In addition, it has been reported that intracellular bacteria promptly escape from

the phagosome of monocytes and proliferate within the cytoplasm leading to cell death (Kubica et al, 2008). Furthermore, macrophages are long-lived in comparison to monocytes and are mobile cells that carry phagocytosed pathogens to the lymphoid tissues (Bellingan et al, 1996). Hence, to study the effect of endocytosed Hb on the growth of intracellular bacteria, we induced the differentiation of U937 monocytes into CD163$^+$ macrophage phenotype using PMA and dexamethasone and used it as a model cell system.

Dexamethasone-differentiated monocytes have been reported to serve as a model system to study Hb-uptake and it has been found that glucocorticoid treatment *in vitro* and *in vivo* shifts monocyte differentiation towards a phenotype with a high Hb clearance and detoxification capacity (Vallelian et al, 2010). To prove that the observed phenotypes were indeed due to Hb uptake and not due to dexamethasone treatment, we had included an experimental control in which the cells were differentiated but not treated with Hb (denoted "unt" for untreated).

**3.2.1.2 CD163-upregulation and Hb uptake by macrophages**

Initially, we optimized the time for endocytosis of Hb by U937-derived macrophages upon incubating the cells with 0.5 mg/ml Hb over a time course of up to 60 min. **Figure 3.29 A** shows maximal co-localization of CD163 and Hb by 45 min. To further confirm this, we performed flow cytometry to verify the endocytosis of Hb at 45 min in CD163$^+$ U937-macrophages (**Figure 3.29 B**).
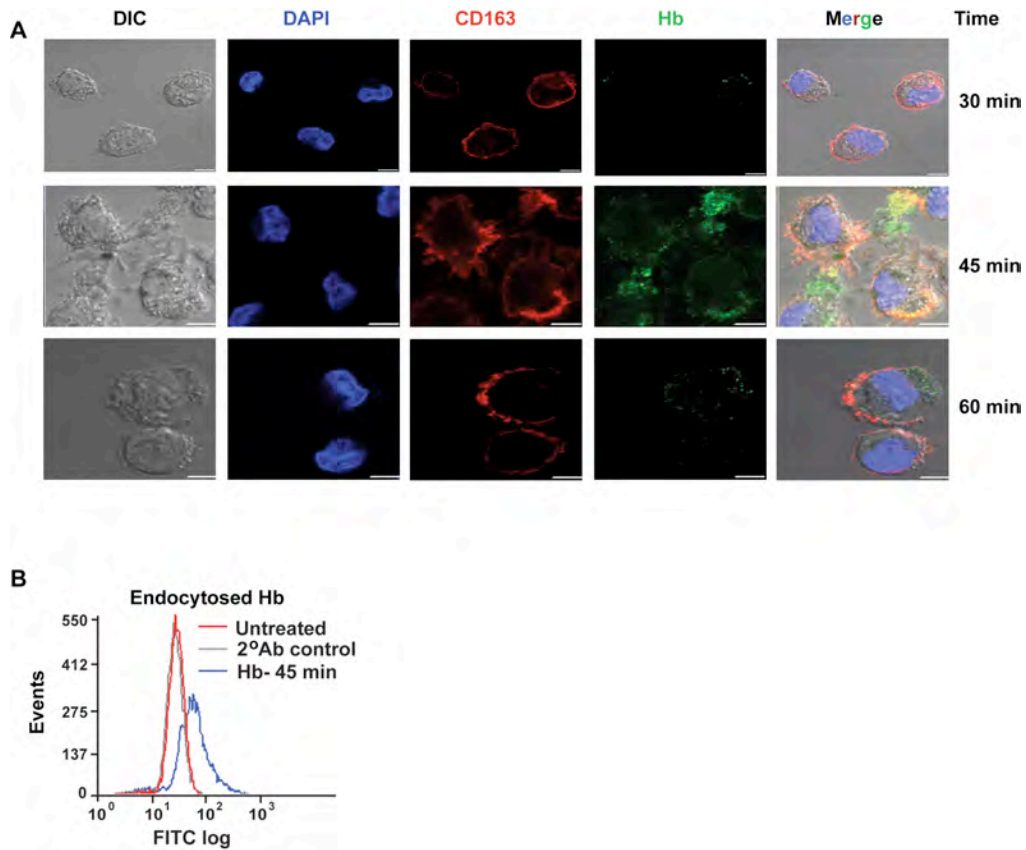
**Figure 3.29: Endocytosis of Hb by CD163^{+} U937-macrophages.** (**A**) Differentiated-U937 macrophages were incubated with 0.5 mg/ml Hb for up to 60 min and immunostained for CD163 and Hb. Co-localization of CD163 and Hb and maximal Hb uptake was observed at 45 min. Images were acquired using the LSM510 META confocal microscope under the 100x oil objective. Scale bars, 5 μm. (**B**) Flow cytometric analysis of Hb-uptake by differentiated U937 macrophages upon incubation with 0.5 mg/ml Hb for 45 min. Untreated refers to cells treated with PBS supplemented with 2% FBS. Cells stained with secondary antibody (2$^{\circ}$ Ab) alone were used as a control to detect non-specific staining.

**3.2.1.3 Infection of Hb-primed macrophages resulted in higher intracellular bacterial load**

To study the effect of the exposure of macrophages to Hb on the survival of intracellular bacteria, we pre-incubated U937-derived macrophages with 0.5 mg/ml Hb for 45 min during which we observed maximal Hb endocytosis (Figure 3.29). The cells were then infected for 30 min with either *Staphylococcus aureus* (gram-positive) or *Salmonella enterica serovar typhimurium* (gram-negative). Since 30 min is usually employed for phagocytosis of *S. aureus* (Garzoni & Kelley, 2009) and *S. typhimurium* (Forsberg et al, 2003) into human macrophages, we used this time period consistently in our experiments. The remaining extracellular bacteria were removed by centrifugation and washing (refer section 2.2.13) and the cells were cultured for a time course of up to 4 h. At each time point, the cells were lysed and the intracellular bacterial load was quantified by colony forming unit (CFU) assay of the cell lysate.

By 4 h post-infection, the Hb-primed macrophages harbored nearly twice the number of intracellular bacteria when compared to unprimed control macrophages, which were also corticosteroid-differentiated, but treated with PBS supplemented with 2% FBS (**Figure 3.30**). Hence, the time-dependent increase in the intracellular bacterial load in Hb-primed macrophages was likely due to the internalized Hb rather than immune suppression by corticosteroids. In addition, this phenomenon is independent of the gram character of the bacteria, because both *S. aureus* and *S. typhimurium* showed similar level of viability inside the U937 cells.
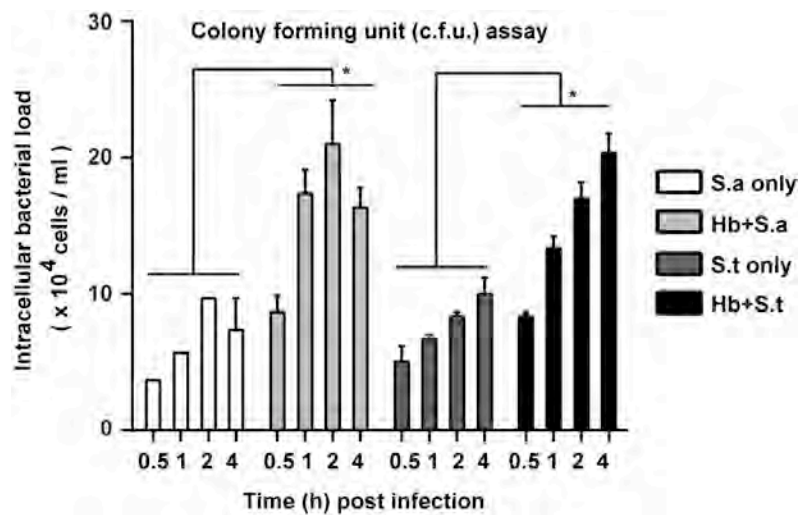
**Figure 3.30: Hb-primed macrophages harbor higher intracellular bacteria upon infection**. U937-dervied macrophages ($0.5 \times 10^6$) were either left untreated or pre-treated with 0.5 mg/ml Hb for 45 min and then infected with *S. aureus* (S.a) or *S. typhimurium* (S.t) at multiplicity of infection (MOI) of 10. The lysates from cells harvested at the indicated time points were serially diluted onto agar plates overnight and the number of colony forming units calculated. * indicates $p < 0.05$.

To verify the physiological significance of this phenomenon, we used confocal microscopy to monitor and quantify the intracellular *S. aureus* and *S. typhimurium* in Hb-primed or unprimed primary monocyte-derived macrophages. The bacteria were stained using the stain BacLight Red stain (refer section 2.2.6.6). In agreement with results shown in Figure 3.30, we consistently observed a time-dependent increase in the mean number of *S. aureus* and *S. typhimurium* per cell after Hb priming (**Figure 3.31 A and B**). Z-stack images were acquired to ensure that only the intracellular bacteria were quantified (**Figure 3.32**). Taken together, our results suggest that the Hb endocytosed by the macrophages supports the intracellular growth and persistence of bacteria during infection.

115
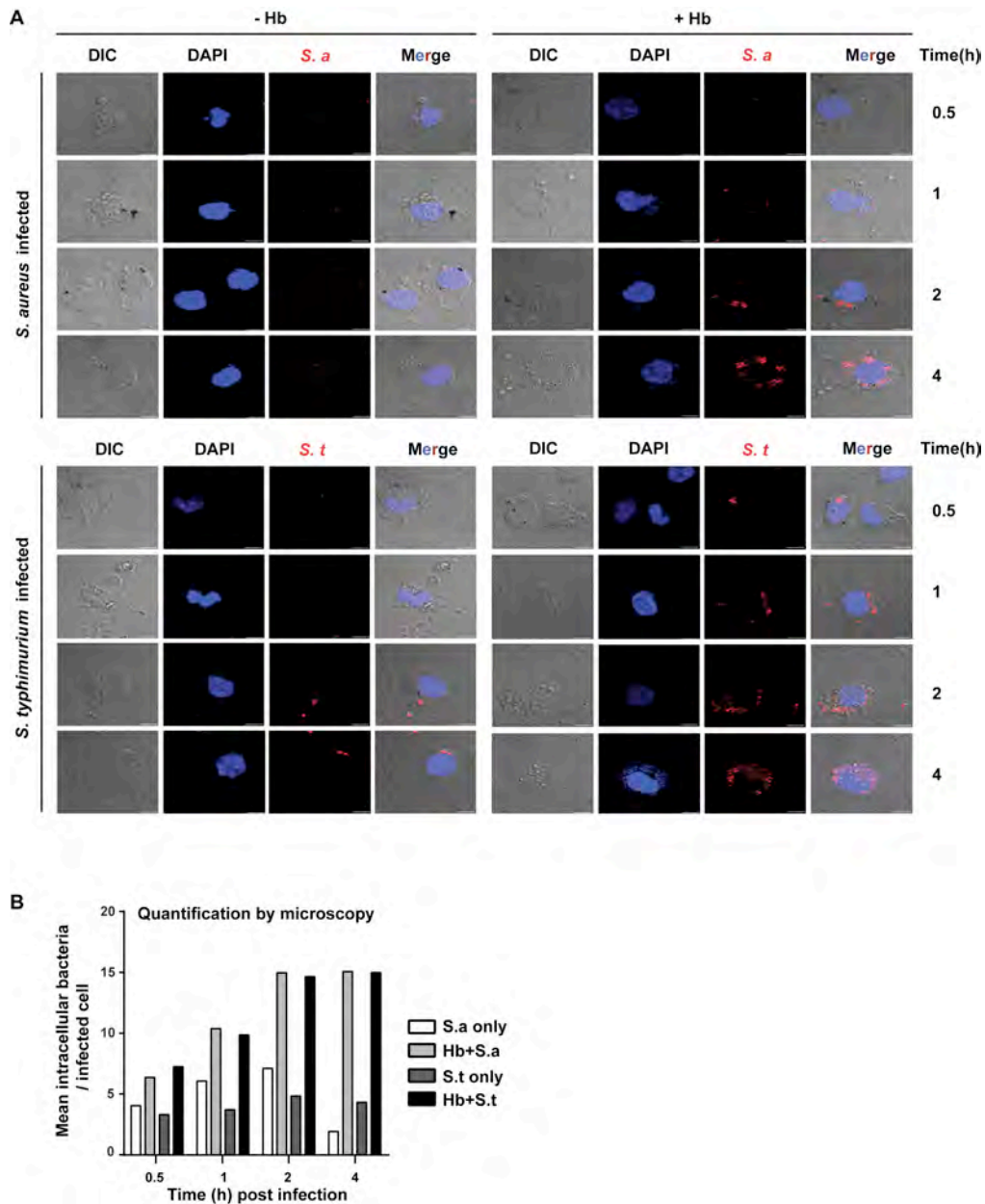
**Figure 3.31: Higher intracellular bacteria in Hb-primed macrophages upon infection.** (**A**) Confocal microscopy to stain the intracellular *S. aureus* or *S. typhimurium* (red) in primary monocyte-derived macrophages at the indicated time points with or without pre-treatment with 0.5 mg/ml Hb for 45 min. Images were acquired using the LSM510 META confocal microscope under the 100x oil immersion objective. Scale bars, 5 µm. (**B**) The mean number of intracellular bacteria per infected macrophage at the indicated time points was quantified by microscopy. In panel B, the mean number of intracellular bacteria was calculated from 3 independent experiments.

116
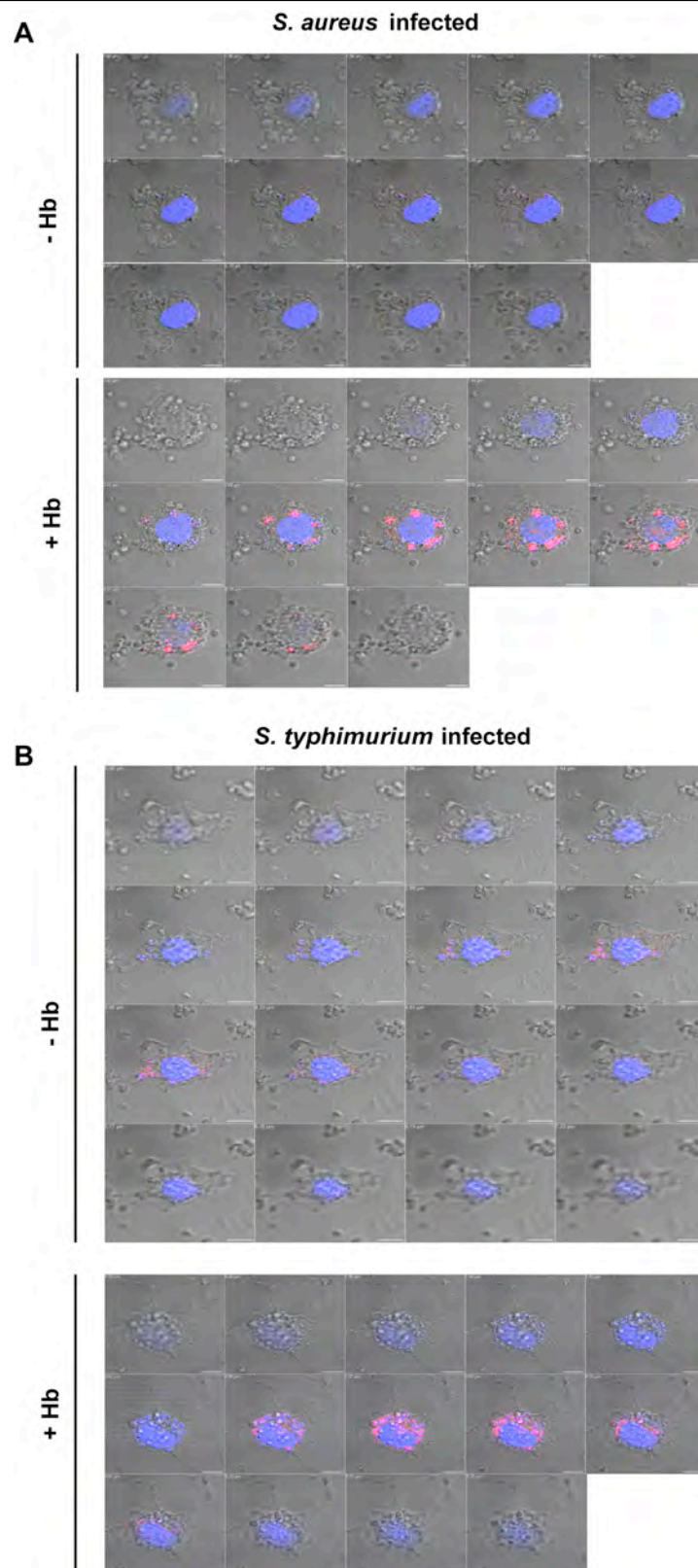
**Figure 3.32: Z-stack images of intracellular *S. aureus* and *S. typhimurium* in macrophages.** Z-stack images, acquired at an interval of 0.48 μm show the intracellular localization of *S. aureus* (S.a) or *S. typhimurium* (S.t) (red) at 4 h post-infection in U937-derived macrophages with or without pre-treatment with 0.5 mg/ml Hb for 45 min. Scale bars, 5 μm.

117

**3.2.2 Intracellular bacteria manipulate iron-responsive signaling molecules in the host**

**3.2.2.1 Upregulation of HO-1 in intracellular bacteria-infected Hb-primed macrophages**

Our previous results showing enhanced intracellular growth of bacteria in Hb-primed macrophages (Figures 3.30 and 3.31) prompted us to examine the regulation of the critical heme-iron responsive signaling molecules by intracellular bacteria. Heme-oxygenase-1 (HO-1) is a heme responsive enzyme that breaks down heme into carbon monoxide, biliverdin and free iron (Tenhunen et al, 1968). **Figure 3.33** shows that Hb treatment upregulates HO-1 in macrophages. Hb-primed macrophages infected with intracellular bacteria (*S. aureus/ S. typhimurium*) showed higher HO-1 expression when compared to unprimed infected control macrophages indicating that the intracellular bacteria might upregulate HO-1 in host cells to detoxify the redox active Hb and to feed on the heme iron liberated. In contrast, Hb-primed macrophages infected with the extracellular bacteria, *P. aeruginosa* showed a drop in the HO-1 expression compared to unprimed infected controls.
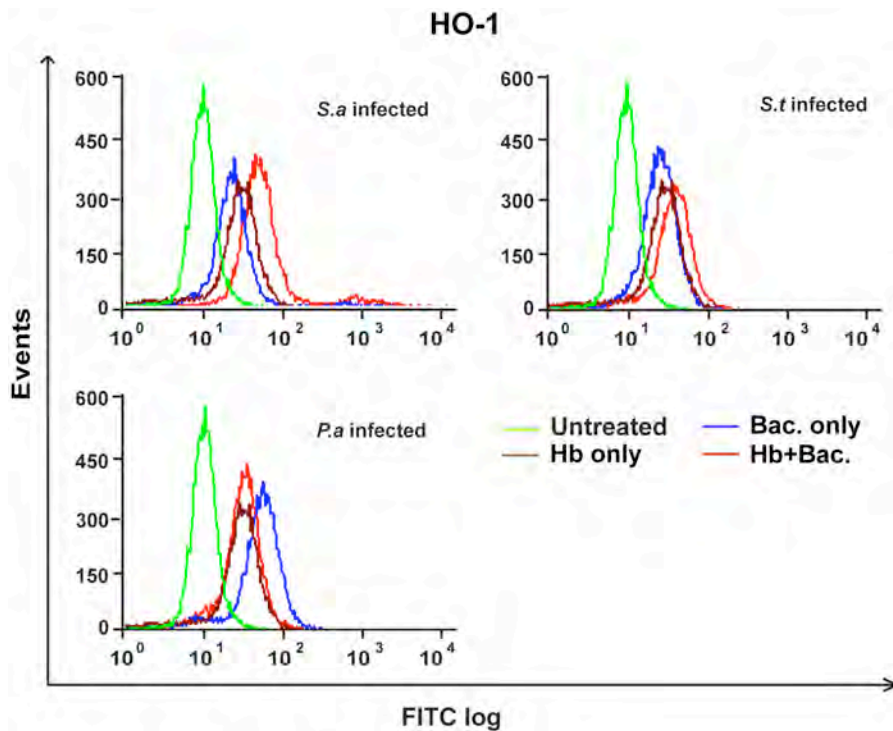
**Figure 3.33: Hb-primed macrophages infected by *S. aureus* or *S. typhimurium* upregulate HO-1.** Flow cytometric analysis showing the peak shift in the histograms of HO-1 staining in Hb-only treated and Hb-pretreated U937-derived macrophages at 4 h post-infection with *S. aureus* (*S.a*) or *S. typhimurium* (*S.t*) or *P. aeruginosa* (*P.a*). Untreated refers to cells treated with PBS supplemented with 2% FBS. Bac. denotes bacteria.

## 3.2.2.2 Downregulation of NRAMP-1 in intracellular bacteria-infected Hb-primed macrophages

NRAMP-1 (Natural resistance associated macrophage protein-1) is a divalent metal-ion transporter, which is involved in iron transport out of the phagosome and confers resistance to intracellular infections by depriving the pathogens of the nutrient iron (Wyllie et al, 2002). Hence, we measured the NRAMP-1 expression upon infection by *S. aureus* and *S. typhimurium* in Hb-primed macrophages using flow cytometry. **Figure 3.34** shows that while Hb treatment upregulated NRAMP-1 expression in macrophages, *S. aureus* and *S. typhimurium* infected Hb-primed macrophages showed downregulation of

119

NRAMP-1 when compared to control unprimed macrophages, which were treated with PBS supplemented with 2% FBS. This suggests that the heme-iron dependent intracellular bacteria might downregulate NRAMP-1 to inhibit iron efflux out of the phagosome and hence retaining the iron for their survival inside the macrophages (**Figure 3.35**). In contrast, *P. aeruginosa* infected Hb-primed macrophages did not show a change in NRAMP-1 expression when compared to control unprimed infected macrophages. This could be likely because *P. aeruginosa* being an extracellular pathogen survives independently of the intracellular source of iron during infection. .
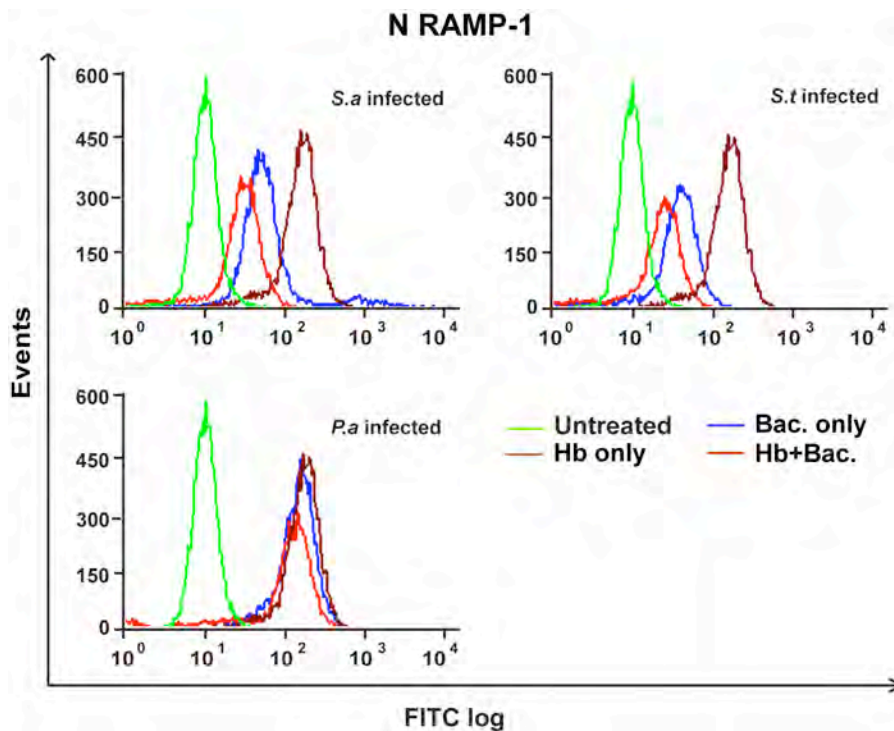


**Figure 3.34: Hb-primed macrophages infected by *S. aureus* or *S. typhimurium* downregulate NRAMP-1.** Flow cytometric analysis showing the peak shift in the histograms of NRAMP-1 staining in Hb-only treated and Hb-pretreated U937-derived macrophages at 4 h post-infection with *S. aureus* (*S.a*) or *S. typhimurium* (*S.t*) or *P. aeruginosa* (*P.a*). Untreated refers to cells treated with PBS supplemented with 2% FBS. Bac. denotes bacteria.
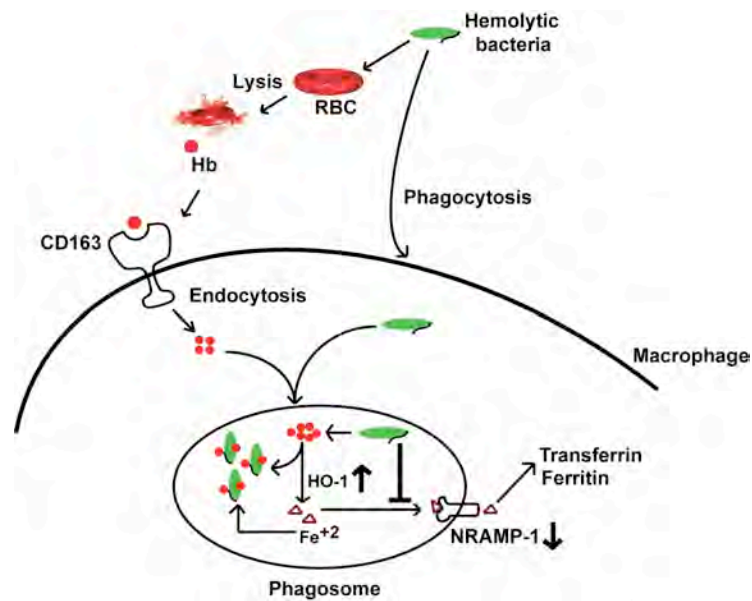
**Figure 3.35: Hypothetical model illustrating how intracellular bacteria might manipulate iron-responsive molecules to survive inside the Hb-primed macrophages.** During a hemolytic infection, free Hb is released from the ruptured RBCs. CD163$^+$ macrophages that uptake extracellular Hb are invaded by intracellular bacteria to utilize the Hb in the phagosome. The intracellular bacteria upregulate the Hb-catabolizing enzyme, HO-1 in macrophages in order to feed on the liberated iron (Fe$^{+2}$). In addition, the bacteria also downregulate NRAMP-1, the iron efflux protein which exports free iron out of the phagosome for subsequent incorporation into iron-chaperone proteins like transferrin and ferritin.

## 3.2.3 Infection of Hb-primed macrophages resulted in downregulation of mitochondrial stress

### 3.2.3.1 Intracellular infection downregulates Hb-ROS production

Redox-active Hb has been reported to aggregate and induce cytotoxicity in macrophages (Kapralov et al, 2009). Since we have shown that intracellular bacteria survived better in Hb-primed macrophages (Figures 3.30 and 3.31), it was pertinent to understand how they modulate Hb-toxicity in macrophages in order to establish survival within the host cell. We measured the mitochondrial

121

ROS production in Hb-treated macrophages, with or without infection by *S. aureus* and *S. typhimurium* at 4 h post-infection, during which we observed maximal intracellular bacteria (Figure 3.30). Stimulation with Hb alone dose-dependently increased mitochondrial ROS production in macrophages when compared to untreated cells, which were incubated with PBS supplemented with 2% FBS (**Figure 3.36**). However, upon subsequent infection of the Hb-primed macrophages with *S. aureus* or *S. typhimurium,* we found that the Hb-induced mitochondrial ROS was significantly reduced compared to control unprimed infected cells, suggesting that the intracellular-resident bacteria actively downregulated or effectively quenched the released ROS.
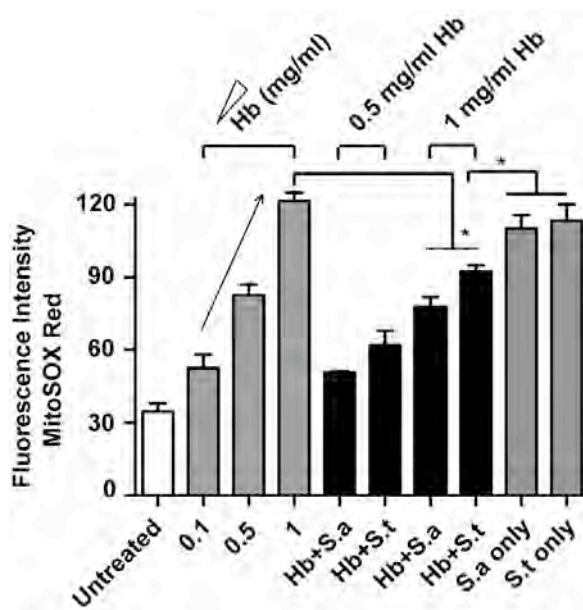


**Figure 3.36: Attenuation of mitochondrial ROS in intracellular bacteria-infected Hb-primed macrophages.** $0.5 \times 10^6$ U937-derived macrophages were loaded with 5 µM MitoSOX Red and either left untreated or pre-treated with 0.5 mg/ml Hb for 45 min. The cells were then infected with *S. aureus* (S.a) or *S. typhimurium* (S.t) at MOI of 10 and the fluorescence intensity of MitoSOX Red was quantitated using the CyAn ADP flow cytometer. Untreated refers to cells treated with PBS supplemented with 2% FBS. * indicates $p<0.05$.

### 3.2.3.2 Intracellular bacteria elicit a "controlled and limited" Hb-POX activation

Next, we examined the *in vitro* activation of the Hb-POX activity by the microbial extracellular proteases secreted into the culture supernatants of *S. aureus* and *S. typhimurium*. In particular, the *S. aureus* strain, PC1839, used in this study expresses the well-characterized extracellular V8 protease (Jiang et al, 2007). However, we did not observe a significant activation of the Hb-POX activity by *S. aureus* PC1839 (**Figure 3.37**), unlike purified proteases like Subtilisin A from *Bacillus subtilis*, which has been reported to significantly induce the Hb-POX activity (Subramanian et al, 2013). This is contrary to expectation since *S. aureus* PC1839 produces high levels of V8 protease, which would have strongly activated Hb-POX activity. Furthermore, *S. typhimurium*, which is also hemolytic (Oscarsson et al, 2002), showed only a weak activation of the Hb-POX activity. In addition, there was no significant dose-dependent effect of the proteases from *S. aureus or S. typhimurium* on the Hb-POX activity. Hence, we speculated that both *S. aureus* and *S. typhimurium* might relegate their proteases in order to utilize the endocytosed Hb in a limited and controlled manner, subtle enough to support their survival without eliciting a strong Hb-POX cycle, which would have generated microbicidal ROS.

123
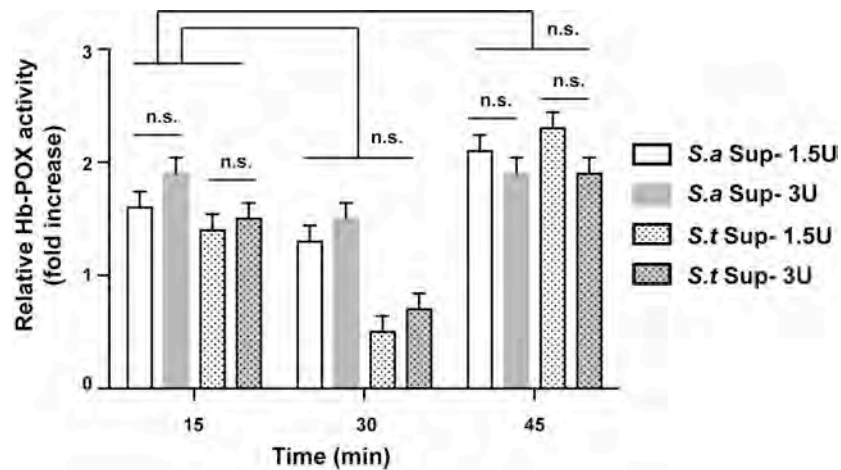
**Figure 3.37: Weak activation of Hb-POX activity by extracellular proteases in the culture supernatants of *S. aureus* or *S. typhimurium.*** Relative POX activity of 10 μg Hb, upon incubation with extracellular proteases in *S. aureus* (S.a)/ *S. typhimurium* (S.t) culture supernatants (Sup) equivalent to 1.5 and 3 units (U) of protease activity over a time course of up to 45 min at 37$^\circ$C. n.s. denotes not significant.

### 3.2.3.3 Intracellular infection partially rescues Hb-induced mitochondrial depolarization and clustering

Next, we examined the effect of Hb-induced ROS production on the mitochondrial pore permeability during infection at 4 h post-infection, on the basis of the interrelationship between ROS and mitochondrial depolarization (Wang et al, 2012). **Figure 3.38** shows that when compared to untreated, Hb stimulation caused a time-dependent decrease in the TMRE dye retention ability, implicating permeabilization of the mitochondria. However, subsequent infection with *S. aureus* or *S. typhimurium* partially rescued the TMRE fluorescence, which is in agreement with the earlier observed reduction in ROS production (Figure 3.36). This consistently suggests that the

intracellular bacteria suppress Hb-POX activity and hence, a reduction in mitochondrial depolarization.
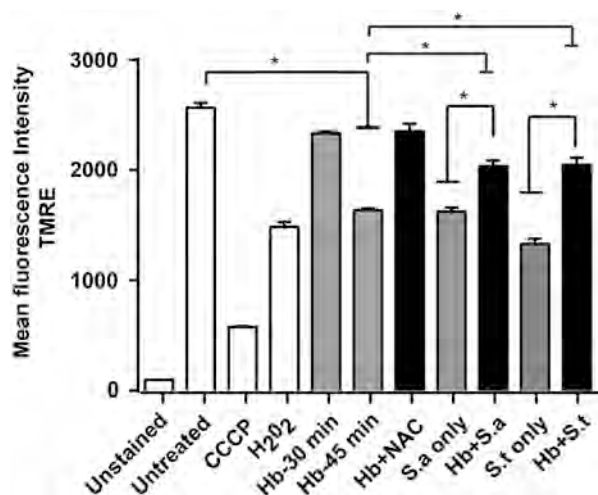


**Figure 3.38: Lower mitochondrial permeabilization in *S. aureus* or *S. typhimurium* infected Hb-primed macrophages.** TMRE staining to measure the mitochondrial pore permeability in U937-macrophages at 4 h post-infection with either *S. aureus* (S.a) or *S. typhimurium* (S.t) at MOI of 10, with or without pretreatment with 0.5 mg/ml Hb for 45 min. 10 µM CCCP and 100 µM $H_2O_2$ treated cells were used as positive controls. Untreated refers to cells treated with PBS supplemented with 2% FBS. * indicates $p < 0.05$.

Coherent with higher ROS production and mitochondrial permeabilization, which occurs during Hb-priming, we also observed mitochondrial clustering in macrophages (**Figure 3.39**, white arrows). Mitochondrial clustering precedes the release of cytochrome c from mitochondria during apoptosis (Haga et al, 2003). ROS has been linked to the clustering of mitochondria during apoptosis (Al-Mehdi et al, 2012) . The clustering phenomenon was abolished when the macrophages were pre-treated with the ROS scavenger, N-acetylcysteine (NAC). Again, *S. aureus* and *S.*

*typhimurium* infection partially reversed the mitochondrial clustering only in Hb-primed macrophages but not in unprimed infected cells. Taken together, the above results suggest that intracellular bacteria downregulate the Hb-induced mitochondrial stress to survive in macrophages.
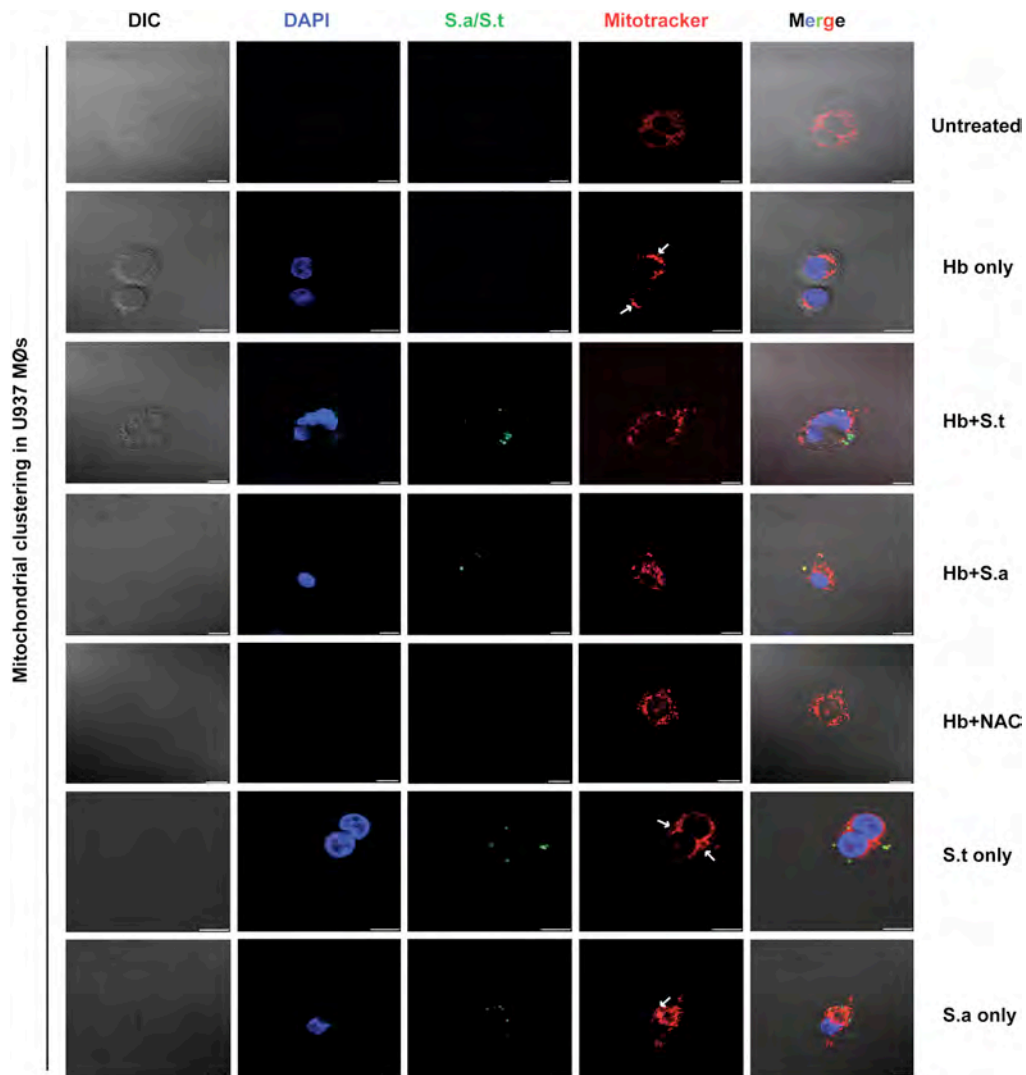


**Figure 3.39: Lesser mitochondrial clustering in Hb-primed macrophages upon intracellular infection.** Mitochondrial staining (red) in Hb-only treated and Hb-pretreated U937-macrophages (Mφ) infected with FITC labeled *S. aureus* (S.a) or *S. typhimurium* (S.t) (green) using 20 nM Mitotracker Orange. White arrows denote mitochondrial clustering. Untreated refers to cells treated with PBS supplemented with 2% FBS. Scale bars, 5 µm.

126

**3.2.4 Intracellular bacteria manipulate host apoptotic signaling pathways**

**3.2.4.1 Downregulation of ERK and p38-induced activation of Bax**

During infection, balance between the pro-apoptotic Bax subfamily and the anti-apoptotic Bcl2-like proteins regulates cell survival (Yang et al, 2002). Bax is a pro-apoptotic subfamily, which translocates to the mitochondria and facilitates the release of cytochrome c, which subsequently induces cell death (refer Figure 1.5). On the contrary, the anti-apoptotic Bcl2 inhibits the activation of Bax and promtes cell survival.

In order to understand how *S. aureus* and *S. typhimurium* might modulate apoptotic signaling in Hb-primed macrophages, we examined the protein levels of Bax and Bcl2 at 4 h post-infection. **Figure 3.40 A** (left panel, red box) shows that while Hb stimulation upregulated the expression of Bax, infection by *S. aureus* and *S. typhimurium* abrogated Bax. In contrast, cells infected without prior Hb-priming showed sustained level of Bax, implying that Hb-priming, which increased the intracellular bacterial count by 2-fold (Figure 3.30), is necessary and sufficient for the infection-induced downregulation of Bax. In contrast to Bax, the level of Bcl2 was maintained in the infected Hb-primed macrophages (Figure 3.40 A), suggesting that the intracellular bacteria might have kept the host cells alive to survive within these cells.

ERK and p38, which belong to the family of mitogen activated protein kinases (MAPKs) are activated by phosphorylation in response to both mitogenic and stress stimuli (Zhuang & Schnellmann, 2006). Previous studies have indicated that signaling via p-ERK and p-p38 mediates apoptosis in

response to stimuli like ROS, infection and are essential for the downstream activation of Bax (Zhuang & Schnellmann, 2006). Hence, we queried whether the intracellular bacteria in Hb-primed macrophages would manipulate the MAPK signaling pathway. **Figures 3.40 A and B** show that while stimulation with Hb alone upregulated both p-p38 and p-ERK signaling, infection of Hb-primed macrophages downregulated both p-p38 and p-ERK. This result is consistent with Bax downregulation and maintenance of Bcl2 level upon infection of Hb-primed macrophages.

### 3.2.4.2 Intracellular infection induces anti-inflammatory cytokine response in Hb-primed macrophages

Since the signaling pathways ultimately culminate in cytokine production, it was pertinent to determine the profiles of pro- and anti-inflammatory cytokines upon infection of Hb-primed cells. We measured TNF-$\alpha$ (pro-inflammatory) and IL-10 (anti-inflammatory) at 6 h post-infection of Hb-primed macrophages. **Figure 3.41** shows that stimulation with Hb alone significantly upregulated the pro-inflammatory cytokine, TNF-$\alpha$, compared to the anti-inflammatory cytokine, IL-10. However on the contrary, infection of the Hb-primed macrophages with *S. aureus* or *S. typhimurium* induced higher IL-10 production, suggesting that the intracellular bacteria might have skewed the cytokine production towards an anti-inflammatory phenotype.
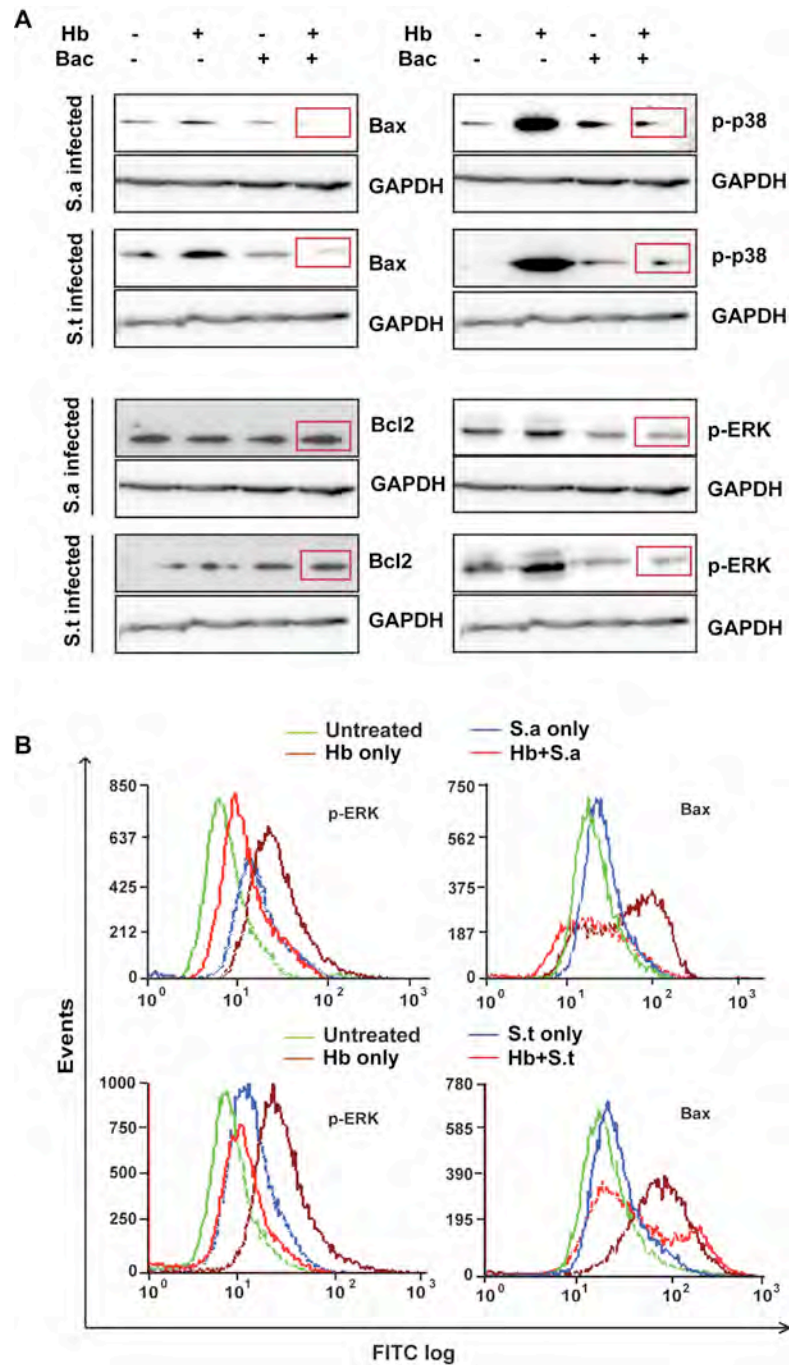
**Figure 3.40: Hb-primed macrophages infected by *S. aureus* or *S. typhimurium* show downregulated MAPK-Bax signaling.** (**A**) Western blotting analysis of p-p38, p-ERK, Bax and Bcl2 in Hb-only treated and Hb-pretreated U937-macrophages infected with *S. aureus* (S.a) or *S. typhimurium* (S.t). Bac. denotes bacteria. Red boxes indicate the downregulation of p-ERK, p-p38 and Bax and maintenance of Bcl2 in infected Hb-pretreated macrophages. GAPDH was used as loading control. (**B**) Flow cytometric analysis showing the peak shift in the histograms of p-ERK and Bax staining in Hb-only treated and Hb-pretreated U937-macrophages infected with *S. aureus* or *S. typhimurium*. Untreated refers to cells treated with PBS supplemented with 2% FBS.
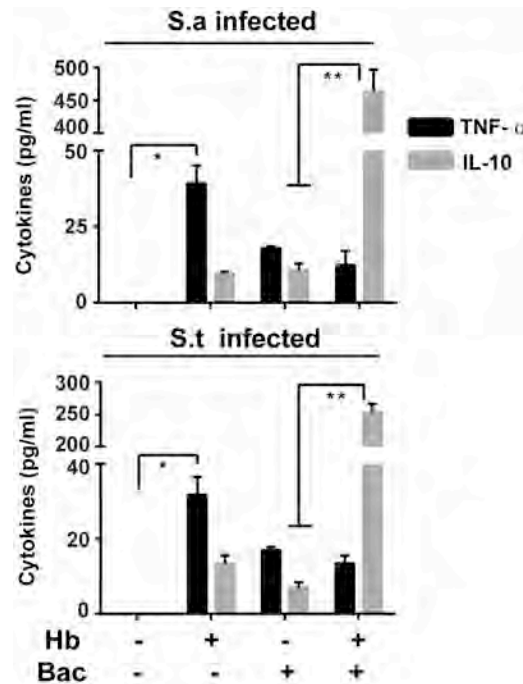
**Figure 3.41: Higher anti-inflammatory cytokine production in intracellular bacteria-infected Hb-primed macrophages.** Cytokine production (TNF-α and IL-10) in Hb-only treated and Hb-pretreated U937-macrophages at 6 h post-infection with *S. aureus* (S.a) or *S. typhimurium* (S.t). * indicates p<0.05; ** indicates p<0.005.

In order to understand how infection by extracellular pathogens impacts the host-cell survival with or without Hb-priming, we used *P. aeruginosa*, a gram-negative extracellular bacterium (Yuan et al, 2012) as a control to study its effect on MAPK-Bax signaling and cytokine production. Contrary to infection by *S. aureus* and *S. typhimurium,* which are known to invade phagocytes, Hb-primed macrophages infected with *P. aeruginosa* showed upregulation of p-ERK and Bax signaling (**Figure 3.42 A)** with concomitantly higher production of the inflammatory TNF-α (**Figure 3.42 B**). Our results suggest that while opportunistic intracellular bacteria such as *S. aureus* and *S. typhimurium* thrive intracellularly within Hb-primed macrophages and keep the host-cells viable, *P. aeruginosa* being an

extracellular pathogen, survives independently of the intracellular source of

Hb during infection.
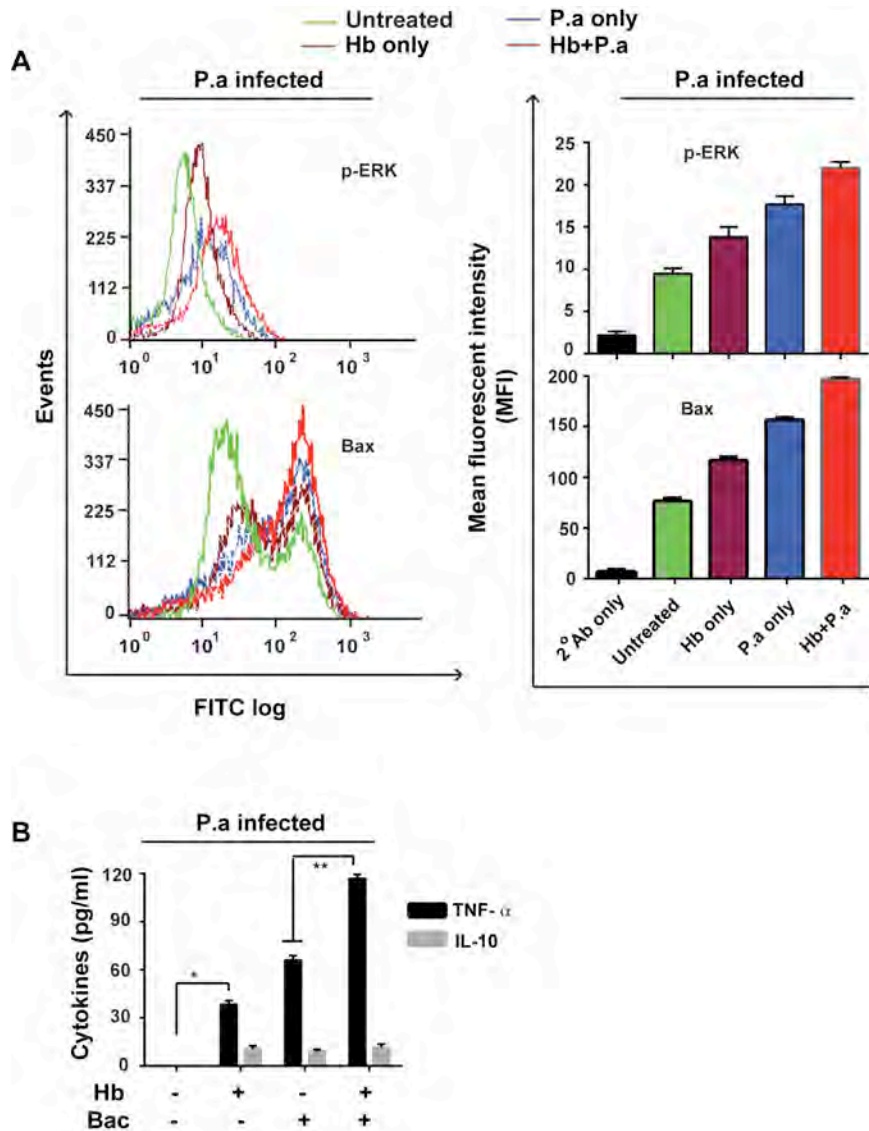


**Figure 3.42:** *P. aeruginosa* **upregulates pro-apoptotic pERK-Bax signaling and inflammatory cytokine production in Hb-primed U937-macrophages.** **(A) Left panel-** Flow cytometric analysis showing the peak shift in the histograms of p-ERK and Bax staining in Hb-only treated and Hb-pretreated U937-macrophages at 4 h post-infection with *P. aeruginosa* (P.a). **Right panel** shows the quantitation of the mean fluorescent intensity of p-ERK and Bax staining. Untreated refers to cells treated with PBS supplemented with 2% FBS. (**B**) Cytokine production (TNF-α and IL-10) in Hb-only treated and Hb-pretreated U937-macrophages at 6 h post-infection with *P. aeruginosa*. * indicates $p<0.05$; ** indicates $p<0.005$.

**3.2.4.3 Supernatant from intracellular bacteria-infected Hb-primed macrophages promotes infectivity of neighboring cells**

To determine whether the anti-inflammatory cytokine production by the intracellular bacteria-infected Hb-primed macrophages would have an impact on the infectivity and viability of 'neighboring' cells, we transferred the conditioned supernatants from unprimed or Hb-primed cells that had been challenged with *S. aureus* or *S. typhimurium* onto fresh recipient cells. Before transfer, the culture supernatants were centrifuged at 10,000xg for 10 min to remove cell debris. To measure the infectivity on the recipient cells, we challenged the Hb-primed recipient cells with *S. aureus* or *S. typhimurium*. Supernatants from Hb-primed cells that had been challenged with *S. aureus* or *S. typhimurium*, promoted the intracellular bacterial growth (**Figure 3.43**). In contrast, control supernatants (from cells treated with Hb alone or *S. aureus/ S. typhimurium* alone or Hb-primed cells that had been challenged with *P. aeruginosa),* did not support intracellular bacterial growth.
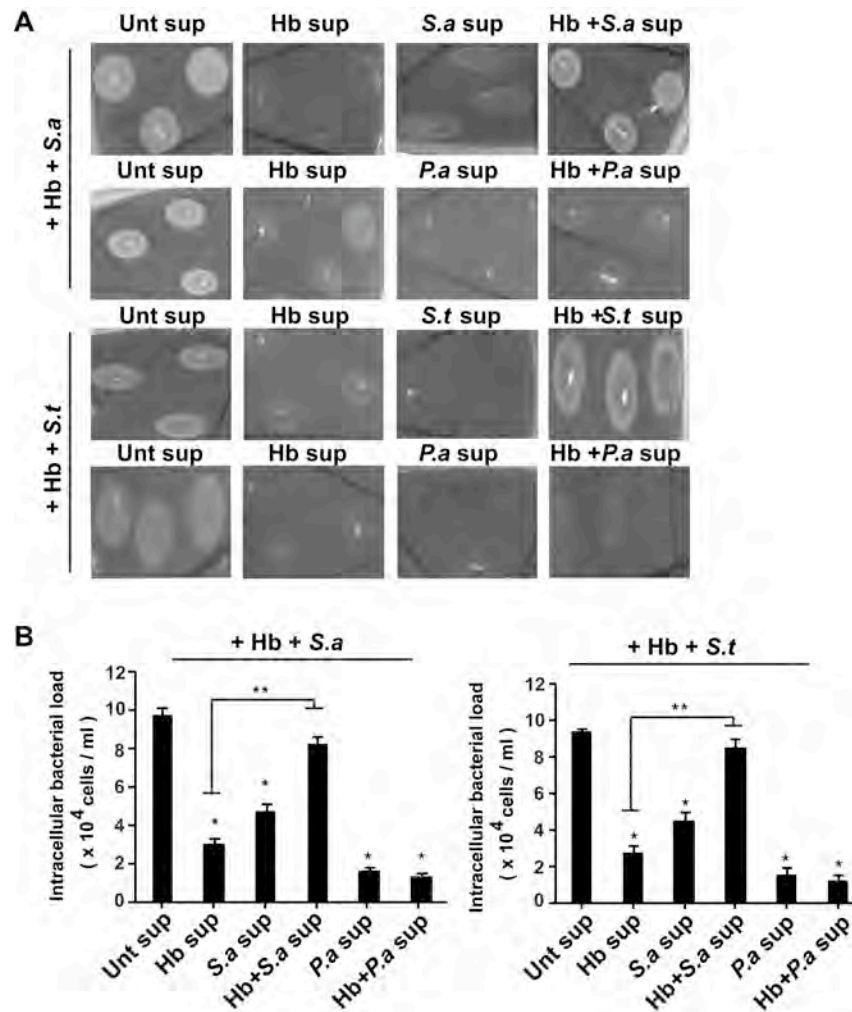
**Figure 3.43: Higher infectivity of recipient cells upon supplementing with supernatants from intracellular bacteria-infected Hb-primed macrophages.** (**A**) CFU assay showing the *S. aureus* (S.a) or *S. typhimurium* (S.t) infectivity of U937-derived macrophages upon supplementing with supernatants (Sup) from macrophages stimulated with either Hb alone or *S. aureus*/ *S. typhimurium*/ *P. aeruginosa* (P.a) alone or Hb-primed cells infected with *S. aureus*/ *S. typhimurium*/ *P. aeruginosa*. (**B**) Intracellular bacterial load with or without supplementing the conditioned supernatants. * indicates $p < 0.05$; ** indicates $p < 0.005$. Untreated (Unt) refers to cells treated with PBS supplemented with 2% FBS.

In agreement with the above finding, we also recorded significantly higher host-cell viability and higher production of anti-inflammatory cytokine, IL-10 by recipient cells upon transfer of supernatants from Hb-primed cells that had been challenged with *S. aureus* or *S. typhimurium*, when compared to

all of the control supernatants (**Figure 3.44 A and B**). Taken together, we speculate that the anti-inflammatory cytokine production induced by intracellular bacterial infection in Hb-primed macrophages, promotes infectivity in 'neighboring' cells while maintaining the host-cell viability.
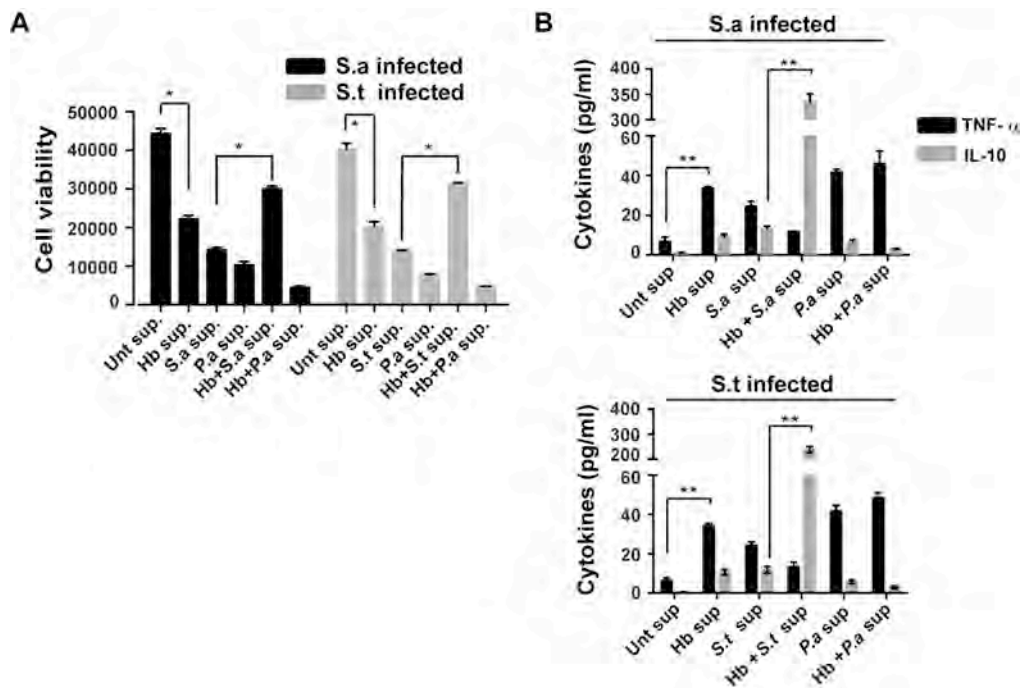


**Figure 3.44: Higher cell-viability and anti-inflammatory cytokine production by recipient cells upon supplementing with supernatants from intracellular bacteria-infected Hb-primed macrophages.** (**A**) Cell viability and (**B**) cytokine production by recipient cells upon transfer of supernatants (sup) from Hb alone treated cells or *S. aureus* (S.a)/ *S. typhimurium* (S.t)/ *P. aeruginosa* (P.a) infected cells with or without Hb priming. Y-axis in panel A indicates the fluorescence (560/590nm) of CellTiter-blue dye. * indicates p<0.05; ** indicates p<0.005. Untreated (Unt) refers to cells treated with PBS supplemented with 2% FBS.

**3.2.5 Hb-primed macrophages harboring intracellular bacteria show suppressed apoptosis**

**3.2.5.1 Intracellular infection partially inhibits apoptosis**

Apoptosis is a mechanism by which an infected-cell commits suicide to prevent the spread of infection to neighboring cells (refer Figure 1.5). Hence to examine the impact of intracellular infection on the host-cell survival, we measured the extent of apoptosis of cells at 4 h post-infection with either intracellular (*S. aureus*/ *S. typhimurium*) or extracellular (*P. aeruginosa*) bacteria, with or without Hb-priming. **Figure 3.45** shows that while treatment with Hb alone induced apoptosis in macrophages, infection of Hb-primed macrophages with *P. aeruginosa* further increased the level of apoptosis compared to that of infected macrophages that were not primed with Hb. This is consistent with our observation of higher activation of the MAPK-Bax signaling pathway and consequently, higher TNF-$\alpha$ production (Figures 3.42). On the contrary, primed macrophages infected with either *S. aureus* or *S. typhimurium* showed a significant drop in the level of apoptosis when compared to unprimed but infected macrophages. This result is concordant with our earlier finding of a drop in the activation of the MAPK-Bax pathway and higher IL-10 production as opposed to TNF-$\alpha$ (Figures 3.40 and 3.41).

135
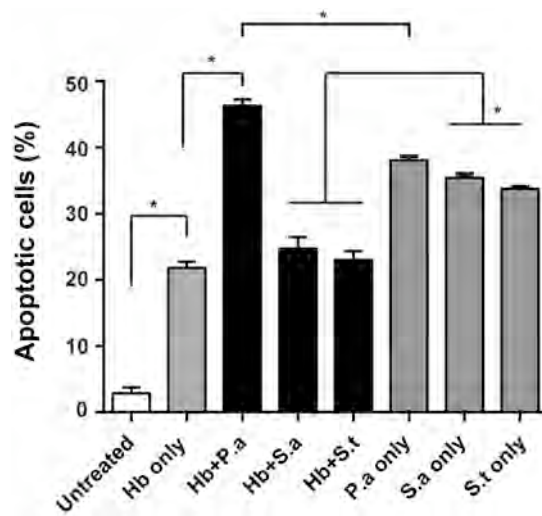
**Figure 3.45: Hb-primed macrophages harboring intracellular bacteria show suppressed apoptosis**. Apoptosis of Hb-only treated and Hb-pretreated U937-macrophages at 4 h post-infection with *P. aeruginosa* (P.a) or *S. aureus* (S.a) or *S. typhimurium* (S.t) was quantified by staining with annexin V-FITC and 7-AAD. Untreated refers to cells treated with PBS supplemented with 2% FBS. * indicates p<0.05. ** indicates p<0.005.

## 3.2.5.2 Intracellular infection blocks activation of caspases

Caspases are cellular proteases that are activated in an apoptotic cell and orchestrate a catastrophic sequence of events leading to nuclear fragmentation and eventually cell-death (refer Figure 1.5). To corroborate that infection by intracellular bacteria suppressed MAPK-Bax signaling (Figure 3.40) and apoptosis (Figure 3.45) in Hb-primed macrophages, we investigated caspase activation. **Figure 3.46 A and B** shows that treatment with Hb alone induces the activation of both the initiator caspase-9 and the executioner caspase-3. However, infection of primed macrophages with either *S. aureus* or *S. typhimurium* reduced the activity of both caspases-9 and -3.

In stark contrast to *S. aureus* and *S. typhimurium*, the extracellular bacterium, *P. aeruginosa* induced higher caspase-9 and sustained caspase-3 activation in Hb-primed macrophages when compared to infected but unprimed macrophages (**Figure 3.47**). This is consistent with our previous results showing higher activation of the ERK-Bax signaling and concomitant production of the inflammatory cytokine, TNF-α by *P. aeruginosa* infected macrophages (Figure 3.42).
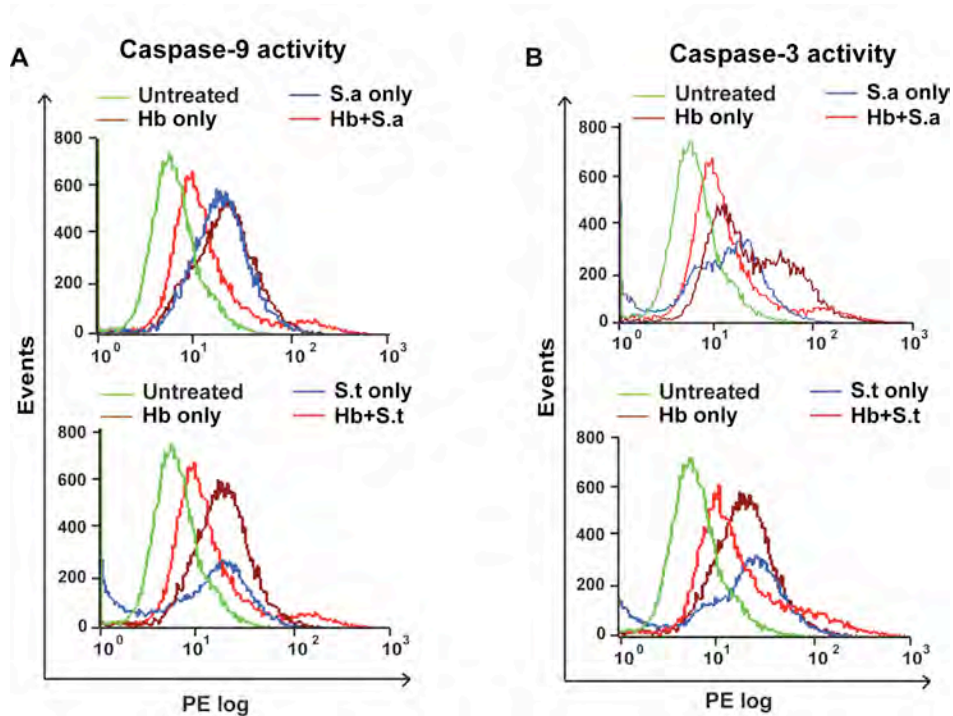


**Figure 3.46: Hb-primed macrophages harboring intracellular bacteria show suppressed caspase activation.** (**A**) Caspase-9 and (**B**) caspase-3 activity in Hb-only treated and Hb-pretreated U937-macrophages infected with *S. aureus* (S.a) or *S. typhimurium* (S.t) was measured by the caspalux and phiphilux assay respectively. Untreated refers to cells treated with PBS supplemented with 2% FBS.
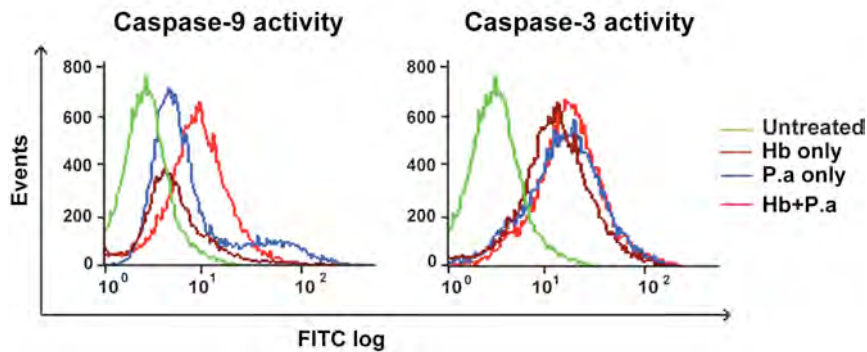
**Figure 3.47:** *P. aeruginosa* **induces higher caspase activation in Hb-primed U937-macrophages.** Caspase-9 and caspase-3 activity in Hb-only treated and Hb-pretreated U937-macrophages infected with *P. aeruginosa* (P.a) was measured by the caspalux and phiphilux assays respectively. Untreated refers to cells treated with PBS supplemented with 2% FBS.

Taken together, in **Section 3.2** we have proposed a molecular mechanism to explain how macrophages, which uptake extracellular Hb during episodes of hemolysis may constitute a silent survival niche for intracellular pathogens. Our findings demonstrate that opportunistic intracellular bacteria such as *Staphylococcus aureus* and *Salmonella typhimurium* preferentially colonize Hb-primed macrophages and modulate the ERK and p-p38 signaling pathways to downregulate apoptosis of the host cells. This has important implications during an infection since intracellular bacteria may use these professional phagocytes as mobile vehicles for dissemination and escape from immune surveillance. This may contribute to the persistence of infections and the ability of pathogens to spread quickly from a local infection to a systemic infection (Garzoni & Kelley, 2009).

Hb exhibits dual characteristics in innate immunity – on one hand, the heme iron is a source of nutrition for the invading microbe and on the other hand, Hb-POX generates microbicidal ROS which is also cytotoxic to the host itself. This presents an unresolved dichotomy during an intracellular infection. It is known that pathogen-mediated proteolysis of host proteins provides a source of amino acids critical for the survival of the microbe (Pishchany & Skaar, 2012). However, we found that intracellular bacteria limit the activation of the Hb-POX activity, to evade the microbicidal ROS production (Figures 3.36 and 3.37) and simultaneously manipulate the vital iron-responsive proteins of the host like HO-1 and NRAMP-1 to detoxify the heme and prevent the efflux of iron out of the phagosome (Figures 3.33 and 3.34) .

Apart from the classical role in cell-proliferation and differentiation, MAPKs (such as p-ERK, p-p38) have also been implicated in the induction of apoptosis both *in-vitro* and *in-vivo* (Cagnol & Chambard, 2010). In particular, p-ERK activation has been associated with cell death induced by ROS (Kralova et al, 2008). We found that Hb-generated ROS induced the activation of p-p38 and p-ERK and consequently, apoptosis of macrophages. However, upon infection of Hb-primed macrophages with intracellular bacteria (represented by *S. aureus* and *S. typhimurium* in this study), we observed a reduction in ERK-induced activation of the pro-apoptotic Bax and downstream caspases while the level of the anti-apoptotic Bcl2 was maintained (Figure 3.40). In stark contrast, infection by extracellular bacteria (*P. aeruginosa*) predominantly upregulated ERK-mediated activation of the pro-apoptotic Bax and caspase-9 (Figures 3.42 and 3.47). Hence, it is possible

that Hb-priming might dictate differential effects on the host-cell survival depending on the nature of infection (intracellular or extracellular bacteria).

Consequent to inhibition of pro-apoptotic signaling, intracellular bacteria also skewed the cytokine profile in Hb-primed macrophages towards an anti-inflammatory IL-10 phenotype rather than pro-inflammatory TNF-$\alpha$ (Figure 3.41), probably to counter apoptosis (Figure 3.45) and promote infectivity in neighboring cells (Figure 3.43). Nevertheless, the suppression of apoptosis by the intracellular bacteria was only partial, likely due to the parallel activation of Toll and Nod-like receptors (Delbridge & O'Riordan, 2007), implicating the potential homeostatic shift towards death when the phagocyte becomes over-burdened with bacteria.

In summary, our findings reveal that intracellular bacteria exploit the Hb-scavenging mechanism to preferentially survive intracellularly within the Hb-primed phagocytes, subtly utilizing the heme-iron without eliciting a strong POX activity to escape from microbicidal ROS. Such intracellular bacteria thrive by downregulating p-ERK- and p-p38-mediated activation of Bax and downstream caspases to silently persist in host cells while suppressing apoptosis (**Figure 3.48**).
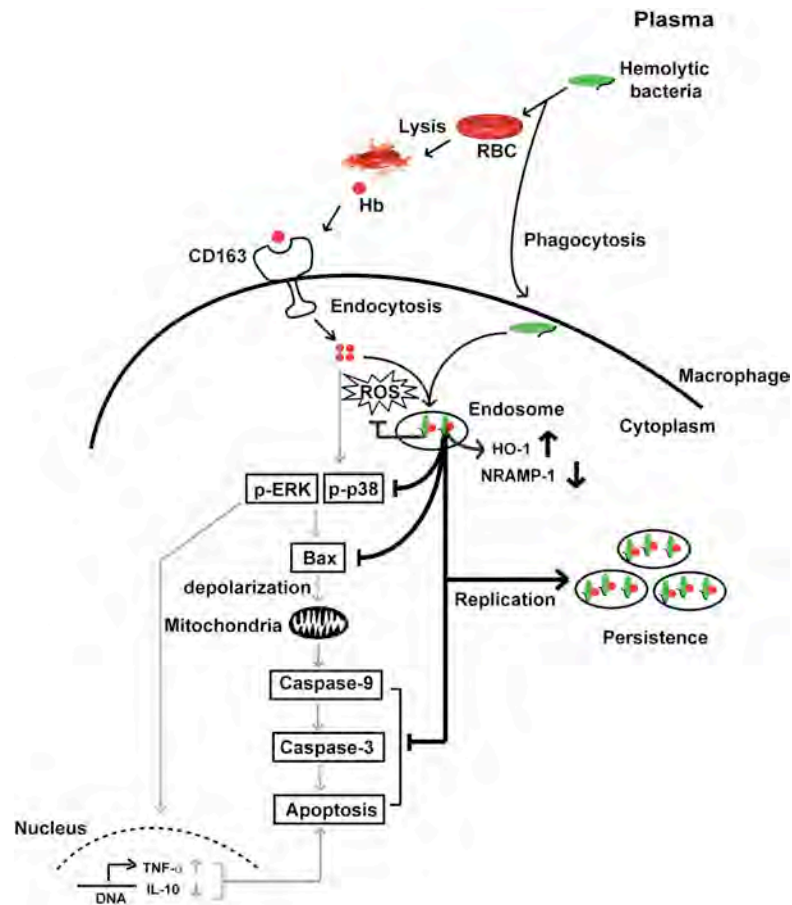
**Figure 3.48: A hypothetical model illustrating the effect of Hb-priming on the susceptibility of macrophages to intracellular infections.** Infection- or injury-mediated hemolysis releases Hb from the ruptured RBCs into the plasma bathing the cells in vasculature. Scavenger receptors expressed on the macrophages such as CD163, uptake and clear Hb from the plasma, thus effectively loading the cells with Hb. Hence, during an infection, it is likely that both Hb and bacteria, which are co-internalized into the macrophages, remain in functional contact with each other in the endosomes. The intracellular bacteria downregulate the redox activity of Hb and at the same time, modulate the iron responsive molecules, HO-1 and NRAMP-1 to utilize the heme-iron for growth and survival. Subsequently, the bacteria downregulate the p-p38 and p-ERK mediated activation of the pro-apoptotic Bax and caspases and skew the cytokine production more towards the anti-inflammatory IL-10 phenotype to silently persist inside the host cells by suppressing apoptosis.

141

# CHAPTER 4
# GENERAL CONCLUSIONS

In this thesis, we explored the innate immune response(s) against extra-erythrocytic Hb. During severe hemolysis induced during tissue injury, trauma or hemolytic infections, Hb reaches extremely high concentrations in the plasma and quickly saturates the major Hb scavenger, Hp. The redox-reactive Hb generates cytotoxic ROS and hence survival depends on the rapid clearance of Hb from circulation. Our results describe a highly efficient two-pass detoxification mechanism involving CD163-mediated endocytosis of Hb into monocytes and endothelial cells in collaboration with plasma IgG and Fc receptors. Such an alternative pathway might become operational when Hp is depleted during severe hemolysis.

Using biochemical, cellular and molecular approaches, we found that mCD163 acts as the frontline receptor for both native and proteolytically activated Hb and directly suppressed the Hb-POX activity on the monocyte membrane. Simultaneously, the mCD163 is proteolytically shed into sCD163, which acts as an additional layer of defense against the cytotoxic Hb in circulation. sCD163 binds to the residual Hb and quickly dampens the redox-reactivity of Hb. Further, we identified that IgG purified from the plasma of healthy individuals, interacts with the sCD163:Hb complex and bridges the sCD163:Hb:IgG complex to FcγR on the monocytes. The internalized Hb is catabolized by HO-1, while the sCD163 is palmitoylated and recycled to the membrane via recycling endosomes to restore the level of CD163 on the monocytes. Interestingly, the sCD163:Hb:IgG complex also transactivated the

vascular endothelial cells, when co-cultured with monocytes and resulted in synergistic cytokine production in response to Hb.

During hemolysis, CD163^{+} macrophages are the major scavenger cells that uptake extracellular Hb. We found that intracellular bacteria preferentially colonize and silently survive within the Hb-loaded macrophages. Importantly, the proteases secreted by the intracellular bacteria elicited a subtle activation of the Hb-POX activity, suggesting that the bacteria might have evolved a "limited Hb-proteolysis" strategy to escape the microbicidal ROS production. In addition, Hb-primed macrophages infected with intracellular bacteria showed upregulation of HO-1 and downregulation of NRAMP-1, implying that the bacteria manipulate iron-responsive proteins in the host to utilize the heme-iron for their survival. Further investigation of the apoptotic signaling pathways and cytokine profiles revealed that intracellular infection of Hb-primed macrophages downregulated the MAPK-Bax signaling pathway, caspase -3 and caspase-9 activation while upregulating the production of the anti-inflammatory cytokine IL-10. Consistently, these results suggested that the intracellular bacteria downregulate pro-apoptotic signaling and inflammatory cytokine production in the host, to ensure their survival.

Overall, we have identified novel Hp-independent mechanism(s) for detoxification and clearance of Hb during severe hemolysis. Our identification of sCD163 as an important acute phase Hb-responsive protein may be applied clinically to subvert Hb-toxicity in patients with acute hemolysis. Moreover, our identification of Hb-primed macrophages as silent survival niche for intracellular bacteria provides new insights into the interrelationship between hemolysis and intracellular bacterial infections.

# CHAPTER 5
# FUTURE PERSPECTIVES

The findings from this thesis open up several new directions for future research. Suitable experiments could be performed to investigate the following research avenues:

**I. sCD163 as a potential biomarker and therapeutic in patients with acute hemolysis**

In this thesis, we have shown that cell-free Hb triggers the shedding of mCD163 from the monocytes to constitute sCD163 in the plasma. Moreover, our findings have revealed that sCD163 in turn binds and attenuates the Hb-POX activity and is itself recycled to restore mCD163 on the monocytes upon endocytosis via the IgG-FcγR pathway. Hence, plasma sCD163 could potentially serve as an early biomarker of hemolysis in patients and possibly protect against the oxidative damage induced by the redox-active Hb. Future experiments could be designed to measure the level of plasma sCD163 using CD163-specific sandwich ELISA in rodent models of hemolysis. Correlation with other early markers of inflammation and sepsis such as C-reactive protein may be examined in patients with hemolytic diseases. This will help to verify the ability of sCD163 to serve as a prognostic marker of hemolysis during inflammation and sepsis. Further, the therapeutic efficacy of sCD163 could be tested in hemolytic models by exogenous administration of sCD163 using osmotic pumps. The formation of sCD163-Hb complexes in the plasma could be analyzed *in vivo* and the effects on Hb clearance and tissue damage could be studied in histological sections. Together, these studies may help reveal the

potential ability of sCD163 to function as a biomarker and therapeutic agent for hemolytic patients.

## II. sCD163 infusion to attenuate toxicity of Hb-based blood substitutes

For the past several decades, scientists have been attempting to develop safer Hb-based blood substitutes for patients requiring blood transfusion. However, the major drawback in the development and administration of safe Hb-derived blood substitutes is due to the intrinsic oxidative nature of free Hb, which induces cellular and tissue damage (Alayash, 2010). The key requirement for Hb to be used in blood substitutes is that it should be able to transport oxygen efficiently with minimal redox reactivity. Here, in this thesis, we have identified sCD163 to attenuate the Hb-POX activity *in vitro*. Our work suggests that it might be possible to control the toxic oxidative reactions of Hb by transfusing sCD163 together or prior to Hb-based blood substitutes. This study may be performed in rodents. Experiments could be designed to measure the *in vivo* toxicity of Hb and the production of pro- and anti-inflammatory cytokines with and without sCD163 infusion. The above studies will help in the development of safer Hb-based blood substitutes.

**III. Verification of the role of CD163$^+$ macrophages as a survival niche for intracellular pathogens *in vivo***

We have found that opportunistic intracellular bacteria such as *Staphylococcus* aureus and *Salmonella typhimurium* preferentially colonize Hb-uptaking CD163$^+$ macrophages. Although we have elucidated the molecular mechanisms involved using *in vitro* and *ex vivo* methods, the role of CD163$^+$ macrophages in homing intracellular pathogens during hemolytic infections needs to be verified *in vivo* in rodent models.

Fluorescently labeled bacteria could be employed to track the localization of hemolytic bacteria in mouse models of hemolytic infection, following which the CD163$^+$ macrophages and monocytes could be isolated and analyzed for intracellular bacterial load and cytokine production. Furthermore, using other intracellular bacteria, a comparative study could be performed to understand whether the mechanism of limited-Hb proteolysis is conserved amongst other intracellular pathogens as well. For example, *Porphyromonas gingivalis* has been shown to persist within oral epithelial cells *in vitro* (Madianos et al, 1996) and express surface proteases to degrade Hb and release heme group containing iron (NM et al, 2003). Taken together, the above studies will help to uncover the relationship between hemolysis and infection.

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**CD163 and IgG Codefend against Cytotoxic Hemoglobin via Autocrine and Paracrine Mechanisms**

Karthik Subramanian, Ruijuan Du, Nguan Soon Tan, Bow Ho and Jeak Ling Ding

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# CD163 and IgG Codefend against Cytotoxic Hemoglobin via Autocrine and Paracrine Mechanisms

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Lysis of RBCs during numerous clinical settings such as severe hemolytic anemia, infection, tissue injury, or blood transfusion releases the endogenous damage-associated molecular pattern, hemoglobin (Hb), into the plasma. The redox-reactive Hb generates cytotoxic reactive oxygen species, disrupting the redox balance and impairing the immune-responsive blood cells. Therefore, it is crucial to understand how the immune system defends against the cytotoxic Hb. We identified a shortcut "capture and quench" mechanism of detoxification of Hb by the monocyte scavenger receptor CD163, independent of the well-known dominant antioxidant, haptoglobin. Our findings support a highly efficient two-pass mechanism of detoxification and clearance of Hb: 1) a direct suppression of Hb-pseudoperoxidase activity by CD163, involving an autocrine loop of CD163 shedding, sequestration of Hb, recycling, and homeostasis of CD163 in human monocytes and 2) paracrine transactivation of endothelial cells by the shedded soluble CD163 (sCD163), which further detoxifies and clears residual Hb. We showed that sCD163 and IgG interact with free Hb in the plasma and subsequently the sCD163-Hb-IgG complex is endocytosed into monocytes via FcγR. The endocytosed sCD163 is recycled to restore the homeostasis of CD163 on the monocyte membrane in an autocrine cycle, whereas the internalized Hb is catabolized. Using ex vivo coculture experiments, we demonstrated that the monocyte-derived sCD163 and IgG shuttle residual plasma Hb into the proximal endothelial cells. These findings suggest that CD163 and IgG collaborate to engage monocytes and endothelial cells in a two-pass detoxification mechanism to mount a systemic defense against Hb-induced oxidative stress. *The Journal of Immunology*, 2013, 190: 5267–5278.

Hemolysis due to tissue injury, trauma (1), or infection by hemolytic microbes (2) ruptures RBCs and releases hemoglobin (Hb) into the plasma. The intrinsic cytotoxicity of the cell-free Hb is well established (3). This is due to the pseudoperoxidase (POX) activity of Hb, which catalyzes the production of free radicals such superoxide anion ($O_2^{.-}$), ferryl Hb, and other reactive derivatives such as hydroxyl radical and hypohalous acid (4).

In an infection, microbial proteases specifically trigger the Hb POX activity, leading to a localized oxidative shock at the site of infection (5, 6). The Hb-induced microbicidal reactive oxygen species (ROS) also damages the host itself when it is not rapidly detoxified and removed from circulation. The interrelationship between ROS and the innate immune system in acute lung injury (7), chronic granulomatous disease (8), hemorrhagic shock, and ischemia (9) has been reported, prompting our systematic analysis of the host defense mechanism(s) against the danger molecule, Hb. Although plasma haptoglobin (Hp) has been reported to bind (10, 11) and mediate the internalization of Hb by monocytes/macrophages via the scavenger receptor membrane-associated CD163 (mCD163) (12, 13), Hp does not alter the reactive properties of the Hb heme group (11, 14). Additionally, neither Hp knockout mice (15, 16) nor humans with anhaptoglobinemia (17) display complete morbidity to hemolysis, suggesting that there are alternative mechanisms of detoxification of Hb. Contrary to the widely accepted mode of clearance of Hb via Hp, a recent study has proposed a possible direct interaction between CD163 and Hb even in the absence of Hp (18). Such Hp-independent clearance mechanism of Hb could be especially crucial during severe hemolysis, when Hp is rapidly bound and exhausted (19). The residual cell-free redox-reactive Hb would have been life-threatening, and yet we survive. Therefore it is conceivable that there are alternative mechanisms of detoxification of Hb even when Hp, the dominant antioxidant of Hb, is depleted. Intriguing questions remain unanswered; for example, although Hb has been shown to be directly recruited by CD163 independent of Hp (18), the functional significance of the CD163-Hb interaction to the redox reactivity of Hb is unknown. Furthermore, during inflammation, mCD163 is proteolytically shed from the monocyte membrane into the plasma, and the soluble CD163 (sCD163) (20–22) reportedly binds the Hb-Hp complex in vitro (23). However, the fate of sCD163 under severe hemolytic conditions (when Hp is depleted) remains unclear. All of these findings prompted us to systematically investigate the innate immune mechanisms regulating cell-free Hb, an important danger-associated molecule. Towards

this goal, we sought to 1) decipher the direct effect of CD163 on the Hb POX activity and the consequential cell survival when Hp is depleted; 2) elucidate the fate of sCD163; and 3) explore the potential crosstalk between monocytes and endothelial cells under severe hemolytic conditions, which is based on reports that monocytes and endothelial cells are activated during hemolysis, rendering the endothelium adhesive to blood cells (24, 25).

Contrary to the current understanding that Hp is the primary antioxidant of Hb, we show that CD163 confers a two-pass Hb detoxification effect. First, mCD163 directly suppresses the POX activity of Hb in situ on the monocyte membrane, independent of Hp. Consequently, CD163 also rescues monocytes from Hb-induced apoptosis. The shedded sCD163 further complexes with residual plasma Hb. The sCD163-Hb complex then interacts with IgG in the plasma. The IgG bridges the sCD163-Hb complex to the FcγR, enabling the endocytosis of the sCD163-Hb-IgG complex. Subsequently, the endocytosed sCD163 is recycled via endosomes to the membrane to restore homeostasis of mCD163 in an autocrine manner, whereas the internalized Hb undergoes detoxification. Second, the sCD163 elicits a paracrine cycle, transactivating the proximal endothelial cells to scavenge and detoxify the cell-free Hb.

## Materials and Methods

All experiments were performed according to the guidelines on ethics and biosafety (Institutional Review Board, reference code NUS-IRB 08-296).

### Reagents, human primary monocytes, and cell cultures

Purified human Hb, subtilisin A, rabbit polyclonal anti-human Hb, rabbit anti-human IgG, and the protein synthesis inhibitor cycloheximide (CHX) were obtained from Sigma-Aldrich. Mouse monoclonal anti-human FcγRI (CD64) and goat polyclonal anti-human CD163 were purchased from R&D Systems. Purified mouse anti-human FcγRIII (CD16) and mouse anti-human FcγRII (CD32) were from BD Pharmingen. Rabbit anti-human heme oxygenase-1 (HO-1) was from Cell Signaling Technology. Mouse monoclonal anti–plasma membrane calcium (PMCA) ATPase was from Thermo Scientific. The endocytosis and recycling inhibitors, chlorpromazine and monensin, respectively, were from Calbiochem.

Histiocytic lymphoma cell line SU-DHL-1 (DSMZ), also described as monocytic M5-type cells, the only human cell line that expresses high levels of CD163 (13), and Jurkat cells, a human T cell lymphoblast cell line, were cultured in 5% $CO_2$ at 37°C in HEPES-buffered RPMI 1640 (Invitrogen) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at a density of $2 \times 10^6$ cells/ml. HEK293T and HepG2 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human dermal microvascular endothelial cells (HMVEC), which expresses FcγRII (CD32) (26), were cultured in EndoGRO-LS complete medium (Millipore) on gelatin-coated flasks. Primary human monocytes were purified from buffy coat by Ficoll-Paque (GE Healthcare) density gradient centrifugation (27) followed by immunomagnetic cell sorting using a human monocyte enrichment kit (StemCell Technologies) according to the manufacturers' instructions.

### Coculture experiments

Confluent HMVEC were washed twice with PBS and incubated with freshly isolated primary monocytes or THP-1 cells at a ratio of 1:1 in PBS for 45 min with or without Hb and prepared for immunostaining. For cytokine assays, the cells were cocultured for 24 h in serum-free RPMI 1640 in the presence or absence of Hb, and the supernatants were collected for ELISA.

### Chemiluminescent-based detection of Hb POX activity and $O_2^{.-}$ production

The generation of free radicals ($O_2^{.-}$) by Hb was monitored by the chemiluminescence of Cypridina luciferin analog (28, 29) using the GloMax 20/20 luminometer (Promega). The relative luminescence units per second specifically measures the dynamics of the generation of $O_2^{.-}$.

### Extraction of native membrane proteins and cytosolic proteins

The native membrane and cytosolic proteins from $2 \times 10^6$ SU-DHL-1 cells or primary monocytes were extracted using a native membrane protein extraction kit (ProteoExtract; Calbiochem) according to the manufacturer's instructions. Briefly, cells were washed twice with ice-cold PBS and incubated for 10 min on ice under gentle agitation with 2 ml ice-cold extraction buffer I supplemented with protease inhibitor mixture. The insoluble material was pelleted by centrifugation at $16,000 \times g$ for 15 min at 4°C and the supernatant enriched in soluble proteins was frozen at −80°C. The cell pellet was then incubated with 1 ml ice-cold extraction buffer II supplemented with protease inhibitor mixture for 30 min on ice, with gentle agitation. The insoluble material was pelleted by centrifugation at $16,000 \times g$ for 15 min at 4°C and the supernatant enriched in membrane proteins was collected and used immediately or frozen at −80°C.

### Cloning and expression of CD163 in HEK293T cells

The full-length human CD163 was cloned into pcDNA3.1A (Invitrogen) and expressed in HEK293T cells. HEK293T cells were seeded and grown overnight on 12-well plates (Nunc) at a density of $4 \times 10^5$ cells/well in DMEM before transfection. The cells were transfected using TurboFect (Fermentas) according to the manufacturer's instructions.

### Measurement of intracellular ROS using CM-$H_2$DCFDA dye

The ROS generated within the monocytes was measured using the cell permeant oxidation-dependent fluorogenic dye CM-$H_2$DCFDA (Invitrogen). SU-DHL-1 cells were plated at $2 \times 10^5$ cells/well onto 24-well plates in phenol red–free RPMI 1640. The cells were washed and resuspended in PBS containing 10 μM CM-$H_2$DCFDA for 30 min in the dark and stimulated with 15 μM Hb with or without pretreatment with 0.1 μg/ml anti-CD163. The fluorescence of the dye at 495 nm was measured using a microplate reader (BioTek).

### Measurement of cell viability and apoptosis

Cell viability was measured using a CellTiter-Blue viability assay kit (Promega) following the manufacturer's instructions. Briefly, HEK293T and HepG2 cells seeded overnight on 96-well plates were stimulated with Hb. CellTiter-Blue was added to each well, and fluorescence was measured (excitation 530 nm, emission 590 nm) after 4 h incubation. The mean fluorescence of triplicate wells was calculated and plotted. Staining of early apoptotic cells was performed using the an annexin V-FITC apoptosis detection kit (eBioscience) and propidium iodide viability staining solutions (eBioscience) according to the manufacturers' instructions. Briefly, primary monocytes were stimulated with 15 μM native Hb or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163. The cells were then washed successively with PBS and 1× binding buffer and resuspended in binding buffer at a density of $1 \times 10^6$ cells/ml. The cells were incubated with FITC-conjugated annexin V (20:1, v/v) for 15 min at room temperature and washed. Propidium iodide was added at a dilution of 1:20 to the cell suspension and immediately analyzed on a CyAn ADP flow cytometer (Dako).

### Flow cytometry

SU-DHL-1 cells ($2 \times 10^6$) were washed twice with PBS and fixed in 4% (w/v) paraformaldehyde for 15 min. The cells were then blocked with 2% BSA for 30 min and washed once with PBS (pH 7.4). Subsequently, the cells were sequentially stained with primary goat anti-CD163 (1:100) and NL-557–conjugated secondary Ab (1:200) (donkey anti-goat; R&D Systems). Then the cells were washed three times with PBS, and $10^4$ cells were acquired and analyzed on the CyAn ADP flow cytometer (Dako).

### Preparation of cell lysate and immunoblotting

Cultured cells were harvested, pelleted, and protein extraction was performed in ice-cold RIPA lysis buffer (Cell Signaling Technology) containing 1 mM PMSF and 1× protease inhibitor mixture (Sigma-Aldrich). Fifty micrograms total proteins was resolved by 10% SDS-PAGE under nonreducing conditions and then electrotransferred to polyvinylidene difluoride membrane in Tris-glycine buffer with 20% methanol. Membranes were probed with a goat polyclonal Ab against CD163 (R&D Systems) followed by rabbit anti-goat HRP-conjugated secondary Ab (Dako). For loading control, blots were probed with a mouse mAb against a plasma membrane housekeeping protein, PMCA ATPase (Thermo Scientific), followed by goat anti-mouse HRP-conjugated secondary Ab (Dako). Bands were visualized with SuperSignal chemiluminescence substrate (Pierce).

## Estimation of sCD163 in culture supernatants

SU-DHL-1 cells, plated at a density of $2 \times 10^6$ cells/well in 24-well plates, were stimulated with 15 μM Hb (Sigma-Aldrich) over a time course. This concentration was chosen in view of its pathophysiological relevance (30). The cells were pelleted at $300 \times g$ for 15 min at 22°C, and the concentration of sCD163 in the cell culture supernatants was measured using a human CD163 ELISA kit (Quantikine; R&D Systems).

## Immunofluorescence microscopy

SU-DHL-1 cells or primary monocytes were seeded at a density of $2 \times 10^5$ cells/well onto poly-lysine (Sigma-Aldrich)–coated coverslips and cultured overnight. The cells were then incubated with sCD163-Hb or sCD163-Hb-IgG complex for the indicated time periods. Subsequently, the cells were fixed using 4% (w/v) paraformaldehyde for 10 min, blocked with 1% BSA in PBS, and incubated with a mixture of primary Abs containing goat polyclonal anti-CD163 (1:200) (R&D Systems) and rabbit anti-Hb (1:500) (Sigma-Aldrich) for 60 min at room temperature. Following three washes with PBS (pH 7.4), the cells were incubated with secondary Ab mixture containing NL-557–conjugated donkey anti-goat (1:200) and Alexa 488–conjugated chicken anti-rabbit (1:400). The cells were then washed three times with PBS and mounted on a slide along with the Prolong Gold antifade mounting reagent containing DAPI (Invitrogen). Confocal imaging of the cells was performed on an LSM 510 META microscope (Zeiss) under a ×100 oil immersion objective using the LSM 510 software.

## ELISA to test for interaction between sCD163 and Hb

Freshly isolated human primary monocytes at $1 \times 10^8$ were washed twice with PBS (pH 7.4) and stimulated with $10^{-8}$ M PMA for 2 h at 37°C. sCD163 was isolated from the culture supernatants. The concentration of the affinity-purified sCD163 was determined using a CD163-specific ELISA. sCD163 (0.1 μg/ml) was immobilized onto microplates (Nunc). Increasing concentrations of Hb in PBS were added and the reaction was incubated for 2 h at 37°C. Bound Hb was detected using 1:1000 mouse anti-Hb (Santa Cruz Biotechnology) and 1:2000 goat anti-mouse HRP (Dako Cytomation). The OD at 405 nm was read.

## Pulldown of Hb-associated protein complexes

FITC (Thermo Scientific) was conjugated to Hb that had been preactivated by partial proteolysis with a typical bacterial serine protease, subtilisin A (1.5 U). For pulldown of the sCD163-Hb complex, 10 μg anti-CD163 (R&D Systems) in TBS (pH 7.5) was conjugated to protein A-Sepharose (GE Healthcare Life Sciences) overnight at 4°C. Unbound Ab was removed by washing twice with TBS and the bound anti-CD163 was cross-linked to Sepharose by incubating for 60 min in cross-linking buffer containing 50 mM dimethyl pimelimidate (Sigma-Aldrich) and 200 mM triethanolamine (pH 8.9). The Sepharose beads were blocked using 100 mM ethanolamine and then incubated with sCD163 and activated Hb-FITC for 60 min at room temperature with two washes between each binding step. Subsequently, after three washes, the bound proteins were eluted with 2.5% acetic acid into tubes containing neutralization buffer of 1 M Tris-HCl (pH 12.0).

Upon identification of the interaction between IgG and Hb-sCD163, we pulled down the sCD163-Hb-IgG complex by incubating protein A-Sepharose with 5 μg IgG (affinity-purified from human serum) at room temperature for 60 min. The unbound Ab was removed by washing twice in TBS. The beads were incubated with 5 μg Hb-FITC and 5 μg sCD163 for 60 min at room temperature with two washes between each binding step. Subsequently, after three washes, the complex of sCD163-Hb-IgG was eluted using 2.5% acetic acid into tubes containing neutralization buffer.

## Purification of sCD163 and IgG by affinity chromatography

For purification of sCD163 from cell culture supernatant, 50 μg anti-CD163 in binding buffer (TBS, pH 7.5) was conjugated to protein A-Sepharose by overnight incubation with rotation at 4°C. Unbound Ab was washed twice with binding buffer and the bound anti-CD163 was cross-linked to Sepharose by incubating for 60 min in a cross-linking buffer (50 mM dimethyl pimelimidate in 200 mM triethanolamine [pH 8.9]). The beads were washed twice and incubated with the culture supernatant for 60 min at room temperature. After three washes, bound sCD163 was eluted using 2.5% acetic acid into tubes containing neutralization buffer (1 M Tris-HCl [pH 12.0]).

For purification of IgG from healthy human serum, protein G-Sepharose (GE Healthcare Life Sciences) was incubated with 5 μl serum (contains

~10 mg/ml IgG) (31) diluted to 400 μl in binding buffer (20 mM sodium phosphate [pH 7.0]) overnight with rotation at 4°C. The beads were washed twice with binding buffer, and the bound IgG was eluted using 0.1 M glycine-HCl [pH 2.7] into tubes containing neutralization buffer (1 M Tris-HCl [pH 12.0]). All experiments were validated using IgG purified from at least three different healthy donors.

## Surface plasmon resonance

The real-time biointeraction between IgG, Hb, and sCD163 was analyzed by surface plasmon resonance using a Biacore 2000 instrument (Biacore International, Uppsala, Sweden). IgG was immobilized on a CM5 chip by amine coupling according to the manufacturer's instructions. Increasing doses of Hb at 0.2–0.8 μM was injected over the IgG-immobilized chip in running buffer of 50 mM Tris, 145 mM NaCl with 2 mM calcium [pH 7.4] at a flow rate of 30 μl/min. Anti-Hb at 5–20 nM was injected to verify the specificity of interaction between Hb and IgG. sCD163 was buffer-exchanged to the same running buffer using Vivaspin columns (Sartorius Stedim Biotech) and 50 μl sCD163 (2.5–10 ng/ml) was injected over the bound Hb. The dissociation was for 180 s at the same flow rate. Regeneration of the chip surface was performed by injection of 0.1 M NaOH until baseline was restored. The binding affinities were calculated using BIAevaluation software, version 4.1 applying the drifting baseline model assuming 1:1 interaction model. Response units were subtracted from BSA/N-acetylglucosamine–immobilized reference flow cells (negative control).

## Silencing of FcγR in primary monocytes

To validate the role of FcγR in the uptake of IgG-Hb-CD163, we silenced all three types of FcγR, that is, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). The CD64 targeting small interfering RNA (siRNA) pool was obtained from Dharmacon (Thermo Scientific), and CD32 and CD16 siRNA duplexes were from OriGene Technologies. Primary monocytes $(2.5 \times 10^6)$ were nucleofected with 2 μg siRNA pool using the Amaxa Nucleofector (human monocyte Nucleofector kit, Nucleofector program Y-001). The oligonucleotide sequence of the siRNA pool used to knockdown the FcγR types in primary monocytes are shown in Table I. Scrambled siRNA pool was used as the negative control. Cells were harvested 48 h after nucleofection. The efficiency of knockdown was analyzed by flow cytometry.

## Biotinylation of soluble CD163 and subcellular tracking

Sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3′-dithiopropionate (sulfo-NHS-S-S-biotin; Pierce) was used for biotinylation of sCD163. Briefly, 2 μg/ml sCD163 was incubated with 20-fold molar excess of sulfo-NHS-SS-biotin at room temperature for 60 min. Excess biotin reagent was removed using ultracentrifugal spin columns (10K Amicon Ultra-0.5), and the biotin-conjugated sCD163 was buffer exchanged to PBS (pH 7.4). The level of biotin incorporated into sCD163 was quantified to be 18 biotin molecules per sCD163 molecule. Subsequently, primary monocytes were incubated with either bitoin-sCD163 alone or as a complex with Hb and IgG for up to 90 min at room temperature. The membrane and cytosolic fractions isolated from cells were captured on anti-CD163–coated 96-well microplates for 2 h at room temperature. The biotin-labeled protein bound on the plate was detected by HRP-streptavidin conjugate (ZyMax Grade; Invitrogen). ABTS substrate enabled the detection of the HRP conjugate and OD at 450 nm was read. Three washes with PBST were carried out between incubations.

## Measurement of HO-1 activity

HO-1 activity assay was performed as described earlier (32). Briefly, 50 μl microsomes from cells stimulated with cell-free Hb was added to 250 μl of a reaction mixture containing 0.1 mM NADPH, 1 mM NADP, 1 mM glucose-6-phosphate, 5 mU glucose-6-phosphate dehydrogenase, 2 mg rat liver cytosol (as a source of bilirubin reductase; prepared according to methods in Ref. 33), 100 mM potassium phosphate buffer (pH 7.4), and 1 mg/ml hemin. The reaction was performed at 37°C in the dark for 1 h. The samples were left in an ice bath to terminate the reaction, and 1 ml chloroform was added. The extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40$ mM$^{-1}$ cm$^{-1}$). The HO-1 activity was expressed as micromoles of bilirubin per milligram of protein per hour.

## Quantification of cytokines by ELISA

The levels of TNF-α, IL-8, and IL-10 in the culture supernatants were measured using commercially available kits (OptEIA human TNF-α, IL-8,

and IL-10 ELISA kits; BD Biosciences) following the manufacturer's instructions.

*Statistical analysis*

Data represent means ± SEM of three independent experiments conducted in triplicate each. A $p$ value <0.05 was considered significant by a paired two-tailed Student $t$ test.

## Results

*CD163 directly detoxifies Hb and rescues cells from Hb-induced apoptosis*

Hb was proteolytically activated with a typical bacterial serine protease, subtilisin A, to mimic an infection-mediated proteolysis (5), which released POX-active fragments of <10 kDa (Supplemental Fig. 1A, boxed), in a dose-responsive manner to subtilisin A. Prolonged reaction time led to excessive proteolysis and loss of the 10-kDa Hb POX fragments. To determine whether CD163 affects the Hb POX activity, we knocked in CD163 into HEK293T cells and then incubated the CD163^{+} HEK293T cells or mock-transfected control cells with activated Hb. The POX activity of the activated Hb was measured by a chemiluminescence assay. Fig. 1A

(box) shows that within 10 min, the CD163^{+} HEK293T cells had reduced the POX activity by ∼80%, whereas the control cells were unresponsive, suggesting that the CD163 effectively blocked the Hb from producing $O_2^{.-}$.

To test whether in situ mCD163 directly inhibits Hb POX, we added increasing doses of the SU-DHL-1 membrane extract (enriched in mCD163) to Hb. We found that the Hb POX activity diminished dose-dependently of the membrane extract, both in the presence and absence of Hp (Fig. 1B, Supplemental Fig. 1B). Incubation with 50 μg SU-DHL-1 membrane extract reduced 80% of the POX activity. Addition of Hp (Hp1-1 isoform) (34) further reduced the POX activity dose-dependently of the membrane extract. Furthermore, when the SU-DHL-1 membrane extract was preincubated with anti-CD163, the inhibition of the POX activity was abrogated dose-dependently of anti-CD163, suggesting that mCD163 directly and specifically downregulates Hb POX activity.

Next, we measured the dynamics of ROS production within the SU-DHL-1 cells when challenged with Hb, with or without preincubation with anti-CD163. Fig. 1C (*left panel*) shows that activated Hb induced ∼75% higher ROS production than did native
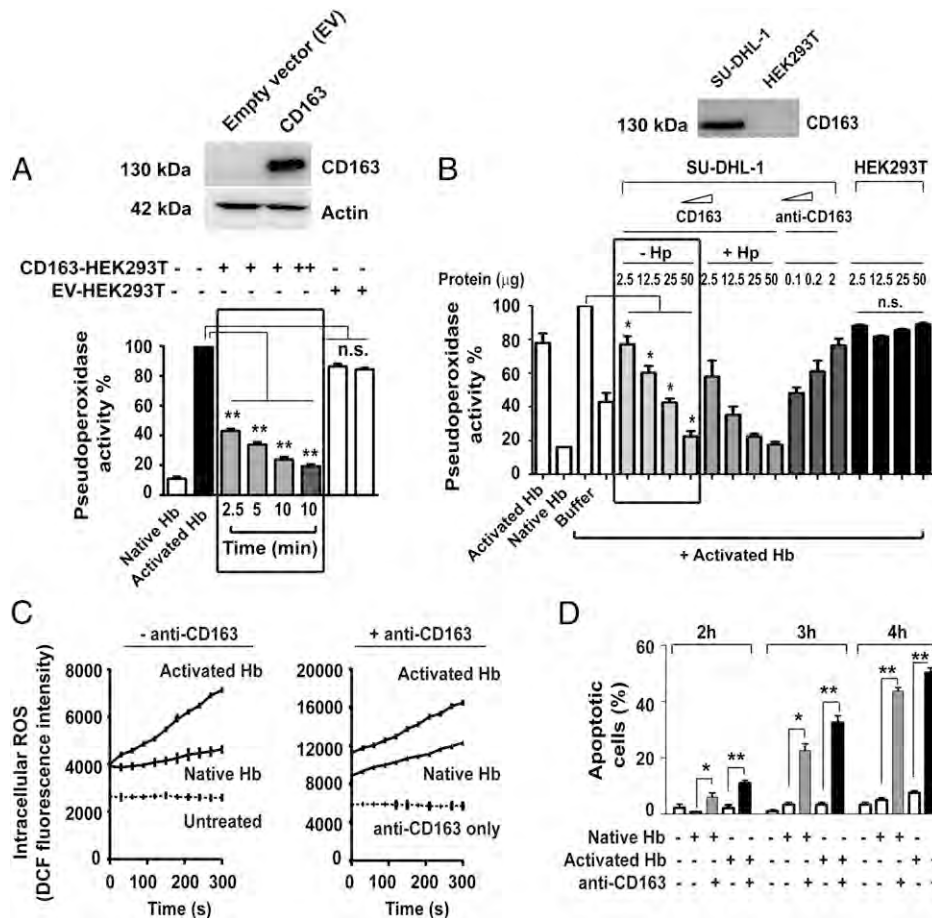


**FIGURE 1.** CD163 directly detoxifies Hb and rescues cells from Hb-induced apoptosis. (**A**) *Top panel*, Western blotting to confirm the knock-in of CD163 into HEK293T cells. *Bottom panel*, Hb POX activity was measured over time of incubation of subtilisin A–activated Hb with 2 × 10^{5} CD163^{+} HEK293T cells or empty vector (EV) only transfected controls. Progressive decrease in Hb POX activity is observed with time and dose (box). ++, Higher dose of CD163^{+} HEK293T cells (10[6] cells). (**B**) *Top panel*, Western blot of the SU-DHL-1 and HEK293T cell membrane extracts probed for CD163. *Bottom panel*, The POX activity of 10 μg activated Hb after incubation for 15 min with increasing doses of the membrane protein extracts of SU-DHL-1 or HEK293T cells with or without pretreatment with anti-CD163. Haptoglobin, Hp1-1 (Hp), was used as a positive control. Progressive decrease in Hb POX activity is observed with increasing dose of CD163 (box). (**C**) Intracellular ROS production in SU-DHL-1 cells incubated with 15 μM native Hb or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163. (**D**) Dynamics of apoptosis in primary monocytes stimulated with 15 μM native Hb or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163. The cells were stained with annexin V-FITC and propidium iodide. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.005$.

Hb. In the presence of functional CD163, the Hb-generated intracellular ROS was halved compared with when CD163 was preblocked using an Ab (Fig. 1C, *right panel*). Furthermore, control cells (HEK293T and HepG2) devoid of CD163 succumbed to Hb, showing increased intracellular ROS and concomitant cell death (Supplemental Fig. 1C), supporting the protective role of CD163 against cytotoxic Hb. Ex vivo real-time quenching activity of Hb POX by CD163 was also observed in primary monocytes (Supplemental Videos 1–7, Supplemental Fig. 1D).

To demonstrate the biological significance of CD163-mediated scavenging and inhibition of Hb redox reactivity, we examined the status of the cell survival/death when the Hb-generated intracellular ROS were allowed to accumulate. Additionally, we queried the consequence of blocking CD163 when plasma Hb reaches concentrations as high as those of severe hemolysis during which Hp is depleted (35). We measured the dynamics of apoptosis when primary monocytes were challenged with Hb with or without blocking of CD163 using Ab. FACS analyses using annexin V-FITC and propidium iodide consistently showed that Hb induced ~50% more apoptosis when CD163 was blocked (Fig. 1D, Supplemental Fig. 1E). Notably, Hb-induced apoptosis was suppressed by the activity of fully functional CD163. Taken together, our findings suggest that CD163 could directly shield monocytes from Hb POX–induced cytotoxicity during a severe hemolysis.

### Hb induces shedding of CD163 from monocyte membrane

Monocytes exposed to inflammatory stimuli are known to shed CD163 (20). To examine the effects of the highly inflammatory Hb

POX on mCD163, we stimulated SU-DHL-1 cells with 15 μM native or proteolytically activated Hb and measured the density of mCD163 on the cells by FACS. We found that the level of mCD163 on the monocytes started to decline within 10 min of stimulation with native Hb, down to ~60% by 1 h but recovered completely within 3–4 h (Fig. 2A, 2B). In contrast, activated Hb induced a more dramatic and steeper drop of mCD163 to ~30%, and the cells recovered only up to 50% of the mCD163 after 4 h. Reciprocal to mCD163, the level of sCD163 in the culture supernatant increased during 60 min (Fig. 2C). Compared to native Hb, the activated Hb induced twice the amount of shedding by 60 min. The Hb-mediated regulation of the level of mCD163 was specific because the housekeeping protein PMCA ATPase remained unaffected (Fig. 2B). To preclude any possible effect of endotoxin contamination on the Hb-induced shedding, both native and activated Hb were tested and found to contain ≤0.05 EU/ml. Consistent with FACS analysis (Fig. 2A) and immunoblotting (Fig. 2B), immunofluorescence microscopy showed fewer CD163^{+} cells at 60 min poststimulation (Fig. 2D). Our data suggest that the monocytes shed mCD163 when they encounter Hb, particularly, the redox active Hb POX.

### sCD163 binds and inhibits Hb POX activity, and the sCD163-Hb complex is internalized by monocytes

Because sCD163 has been shown to bind Hb-Hp complex in vitro (23), we queried whether sCD163 could still bind to Hb when Hp is depleted under conditions of severe hemolysis. We showed that Hb bound directly and dose-dependently to sCD163 in the absence of Hp (Fig. 3A), with the activated Hb binding more



**FIGURE 2.** Hb induces shedding of CD163 from monocyte membrane. (**A**) FACS analysis of mCD163 density on SU-DHL-1 cells treated with 15 μM native Hb or activated Hb during 4 h. Data were normalized against a plasma membrane–localized housekeeping protein, PMCA ATPase. BSA (15 μM)-treated cells served as negative control. *$p < 0.05$, **$p < 0.005$ compared with untreated control. (**B**) Western blot analysis of mCD163 and PMCA ATPase (loading control) in membrane extracts of SU-DHL-1 cells treated with 15 μM Hb during 4 h. (**C**) sCD163 in the culture supernatant was measured using a human CD163-specific sandwich ELISA. Data were normalized against untreated cells. *$p < 0.05$. (**D**) Immunofluorescence analysis of mCD163 on cells treated with 15 μM native Hb or activated Hb for up to 60 min. Scale bars, 10 μm. Images were acquired using Axio Observer Z1 fluorescence microscope (Zeiss) under ×32 air objective and are representative of three independent experiments.
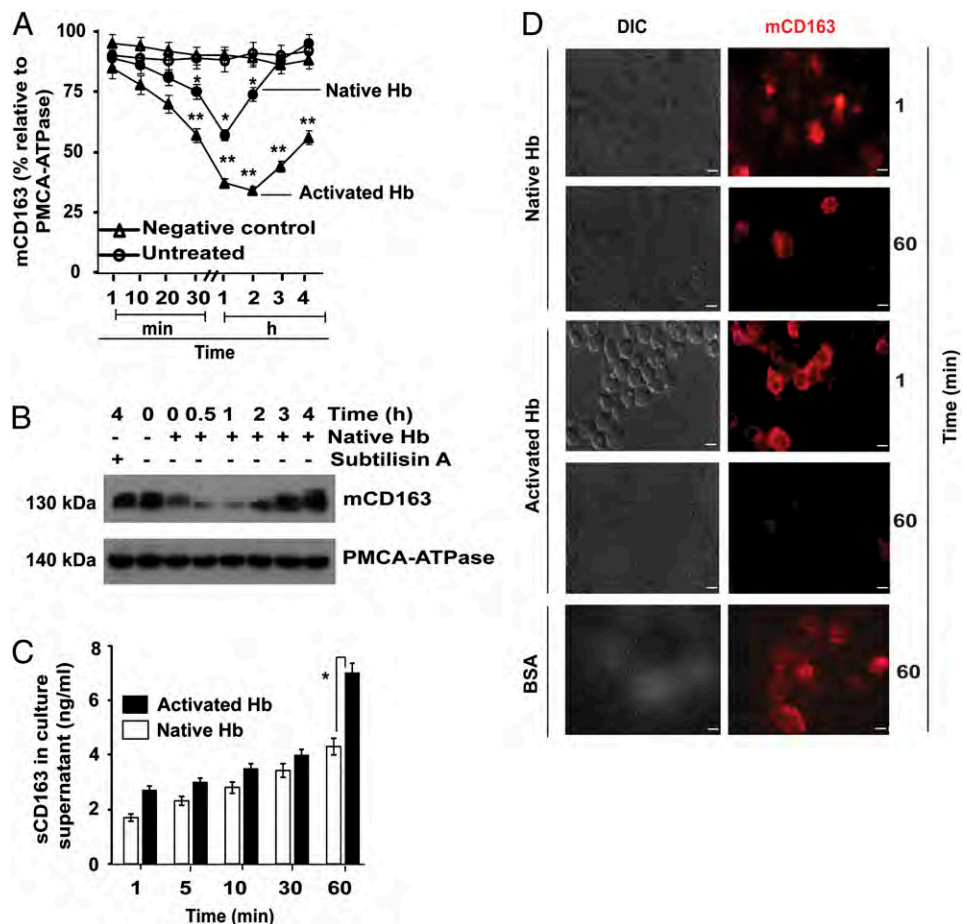
strongly than native Hb. Coimmunoprecipitation studies confirmed the specific interaction between sCD163 and Hb (Supplemental Fig. 2A). Redox-active extracellular Hb was reported to

aggregate and induce cytotoxicity (36), hence necessitating the rapid inhibition of Hb POX even before its uptake into cells. This prompted us to investigate whether binding of sCD163 to Hb
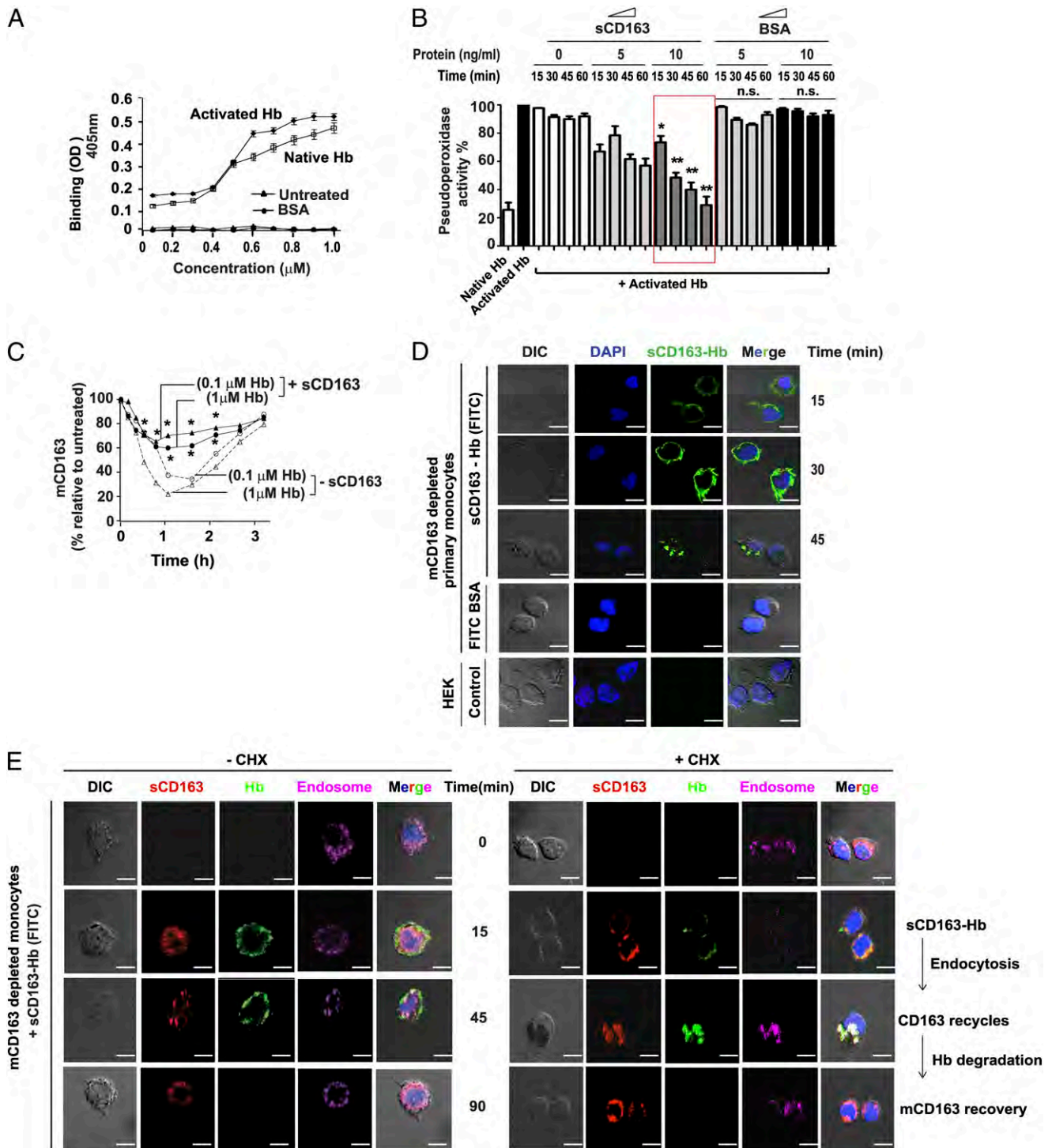
**FIGURE 3.** sCD163 binds and quenches Hb POX, and the sCD163-Hb complex is internalized by monocytes. (**A**) ELISA shows dose-dependent interaction between sCD163 and native or activated Hb (0–1 μM) when 0.1 μg/ml sCD163 was immobilized onto microplates. (**B**) POX activity of 10 μg activated Hb incubated with sCD163 or BSA (0–10 ng/ml) during 60 min. Red box indicates progressive decrease in Hb POX activity induced by 10 ng/ml sCD163. *$p < 0.05$, **$p < 0.005$ compared with untreated control. (**C**) FACS analysis shows dose-dependent effect of activated Hb on mCD163 over time in the presence or absence of 10 ng/ml sCD163. *$p < 0.05$ compared with 0 ng/ml sCD163 controls. (**D**) Purified sCD163-FITC–activated Hb complex (1.5 μM) (green) was incubated for 15–45 min with primary monocytes predepleted of mCD163 and tracked by confocal microscopy. (**E**) mCD163 predepleted monocytes were incubated with 1.5 μM sCD163-FITC–activated Hb complex and 10 μg/ml Alexa 647-transferrin (early and recycling endosomal marker) for up to 90 min with or without 5 μg/ml CHX pretreatment for 60 min. The localization of sCD163 and Hb was tracked by immunostaining. Images were obtained using the LSM 510 META confocal microscope under ×100 oil objective. Scale bars in (D) and (E), 5 μm. Images are representative of three independent experiments using primary monocytes from a single healthy donor.

could affect the Hb POX activity extracellularly. We found that the Hb POX activity decreased significantly and dose-dependently of sCD163, correlating with reaction time (Fig. 3B, box). Within 60 min, up to 70% of the POX activity was suppressed in the presence of 10 ng/ml sCD163, whereas the control protein, BSA, had no effect on the POX activity, confirming the specificity of sCD163 toward Hb.

To query whether sCD163 would influence the level of mCD163 when the monocytes encounter activated cell-free Hb, we supplemented the cells with 0, 5, and 10 ng/ml purified sCD163 followed by stimulation with 0.1 or 1 μM activated Hb. Flow cytometry results indicated that the presence of sCD163 dose-dependently reduced the Hb-triggered shedding of mCD163 (Fig. 3C, Supplemental Fig. 2B, 2C). This suggests that sCD163 exerts a negative feedback on Hb-induced shedding of mCD163, implicating a protective role of sCD163 on mCD163, possibly to maintain the level of mCD163 while sequestering Hb.

Pathogens have evolved efficient heme scavenging strategies to usurp iron from the host hemoproteins (37). Because sCD163 appears to influence the level of mCD163, we hypothesized that the sCD163-Hb complex might be recruited back to the monocyte while simultaneously sequestering the heme iron from the microbial invaders. To test our hypothesis, we studied the fate of sCD163 by incubating the complex of sCD163-FITC–conjugated Hb (activated form) with primary monocytes, which had been depleted of mCD163 (Supplemental Fig. 3A, top panel). The fate of sCD163-FITC-Hb complex on and/or in the mCD163-deficient monocytes was tracked by confocal microscopy. We found that the complex was recruited to the cell membrane within 15 min (Fig. 3D) and internalized by 45 min. However, sCD163 by itself or sCD163-FITC-Hb complex, in the absence of serum, did not bind to cells (Supplemental Fig. 3A, bottom panel), suggesting the potential involvement of serum proteins in trafficking the sCD163-Hb complex into the monocytes. The internalized sCD163 was colocalized intracellularly with transferrin, an early recycling endosome marker (38, 39) (Fig. 3E, left panel). By 90 min, the sCD163 reappeared on the cell membrane, which is consistent with the time at which the Hb-treated monocytes started to recover mCD163 (Fig. 2). To examine whether CD163 from the recruited complex of sCD163-Hb reappeared as mCD163 or whether the restored level of mCD163 arose from new protein synthesis, we applied the protein synthesis inhibitor CHX (40) to the cells prior to treatment with the sCD163-Hb complex. Fig. 3E (right panel) shows that CHX treatment did not compromise the recovery of mCD163, indicating that the mCD163 level was not attributable to de novo protein synthesis, but rather, it likely originated from the internalized sCD163.

### FcγR facilitates the endocytosis of the sCD163-Hb-IgG complex into monocytes

To identify the potential receptor involved in the recruitment of the sCD163-Hb complex into monocytes, we tested the possible role of FcγR because it mediates uptake of oxidized protein complexes from the plasma (41). This prompted us to examine the potential role of IgG, the known ligand of FcγR, which might participate in the sCD163-Hb interactome. We found that indeed Hb in the sCD163-Hb complex coimmunoprecipitated with IgG from the serum of healthy individuals (Supplemental Fig. 3B). The ELISA results corroborated and established a dose-dependent interaction between the sCD163-FITC-Hb complex and the immobilized IgG (Fig. 4A). No binding occurred with FITC-BSA control, suggesting that Hb but not sCD163 in the Hb-sCD163 complex binds to IgG. Furthermore, in the absence of sCD163, Hb displayed reduced affinity for IgG. Because sCD163 binds

Hb (Fig. 3), we sought to test whether purified IgG, Hb, and sCD163 would form a complex in vitro. Real-time biointeraction using surface plasmon resonance analysis showed strong binding between IgG and Hb ($K_D = 1.15 \times 10^{-7}$ M) and between IgG, Hb, and sCD163 ($K_D = 2.25 \times 10^{-9}$ M), producing shift and supershift, respectively, in a dose-dependent manner when the proteins were injected successively onto the IgG-immobilized chip (Fig. 4B, 4C). The specificity of the interaction between Hb and IgG was affirmed by the supershift produced by anti-Hb (Supplemental Fig. 3C).

To investigate whether the sCD163-Hb-IgG complex was endocytosed via interaction with FcγR on the primary monocytes, we performed flow cytometry after incubation with increasing doses of purified complex of sCD163, FITC-Hb, and IgG with wild-type cells and FcγR knockdown cells. The efficiency of knockdown of all three types of FcγRs, that is, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) by the respective siRNA pool (Table I), was verified by the loss of protein at 48 h after nucleofection (Supplemental Fig. 3D). The sCD163-Hb-IgG complex was readily endocytosed by wild-type primary monocytes in a dose-dependent manner (Fig. 4D, top panel). However, the cells knocked down of CD64, CD32, or CD16 showed substantially reduced endocytosis of the sCD163-Hb-IgG complex (Fig. 4D, bottom panel). CD64 knockdown, in particular, compromised the binding of the sCD163-Hb-IgG complex to the greatest extent when compared with CD32 or CD16 knockdown. This could probably be due to the higher affinity of CD64 toward IgG compared with CD32 or CD16 (42). Triple knockdown of all the FcγR types almost completely abrogated the binding of the sCD163-Hb-IgG complex to the cells. The negative controls, BSA, sCD163, sCD163-IgG, and Hb-IgG did not bind to the cells, indicating that the sCD163-Hb complex was specifically endocytosed via interaction with IgG, the ligand that bridges the sCD163-Hb complex to FcγR on the monocyte.

### Endocytosed sCD163 is recycled to mCD163 whereas the internalized Hb is catabolized

Next, we quantified and tracked the subcellular localization of CD163 after endocytosis of the sCD163-Hb-IgG complex into primary monocytes. The monocytes were predepleted of mCD163 followed by treatment with CHX to block subsequent de novo synthesis of CD163. Results showed that within 15 min, CD163 was detected in the membrane fraction, indicative of binding of the sCD163-Hb-IgG complex to the membrane (Fig. 5A). Within 30–45 min, CD163 was localized in the cytoplasm, corroborating the endocytosis of the sCD163-Hb-IgG complex, and this was effectively blocked by pretreatment with chlorpromazine, an inhibitor of endocytosis (43). By 90 min, the internalized CD163 reappeared on the membrane and this was abolished when the cells were pretreated with monensin, a known inhibitor of recycling endosomes (44). When the cells were simultaneously pretreated with both chlorpromazine and monensin, CD163 was only observed on the cell membrane throughout the 90 min duration, indicating that both the endocytosis of the sCD163-Hb-IgG complex and subsequent recycling of the endocytosed CD163 were compromised.

To validate the recycling of sCD163 into mCD163, we incubated primary monocytes with either biotinylated sCD163 alone or as a preformed complex of biotin sCD163-Hb-IgG and tracked the subcellular localization of sCD163 using streptavidin-HRP. The purity of the membrane/cytosol fractions was assessed using membrane (CD64) or cytosolic (tubulin) markers (Fig. 5B, top panel). By 15 min, sCD163 was detected in the membrane fraction and it was endocytosed within 30–45 min (Fig. 5B, bottom panel). By 90 min, sCD163 reappeared on the membrane, consistent with Fig. 5A, validating that sCD163 from the endocytosed
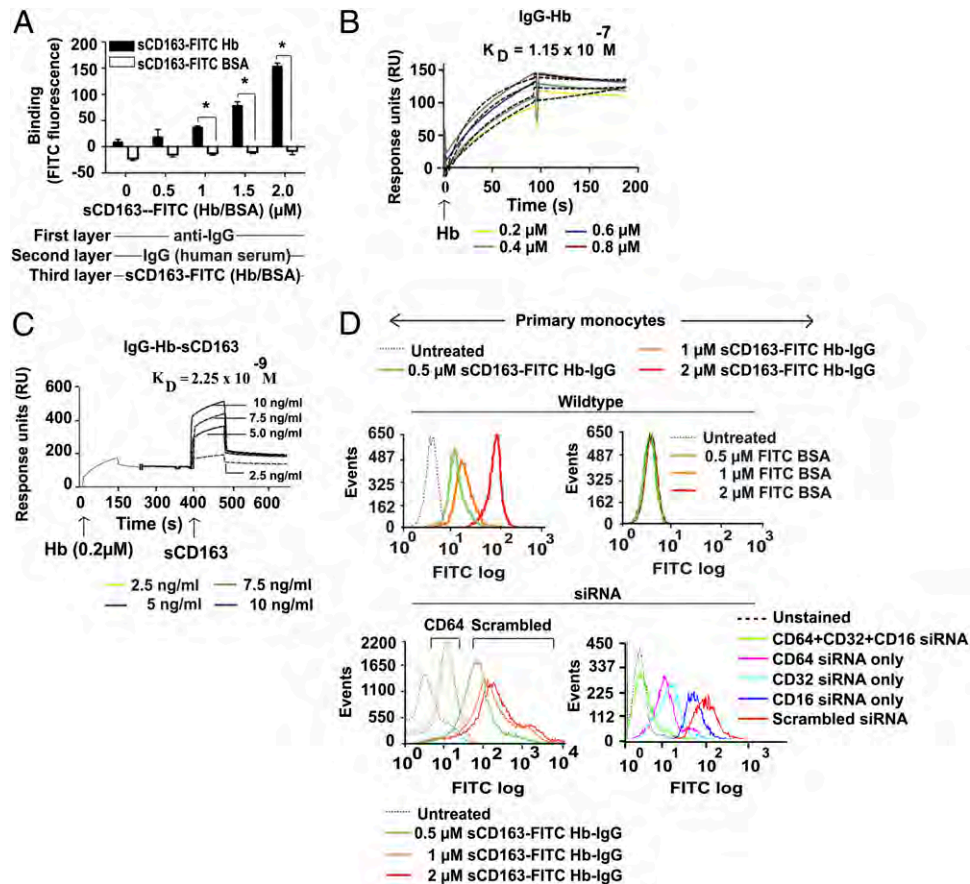
**FIGURE 4.** FcγR facilitates the endocytosis of the sCD163-Hb-IgG complex into monocytes. (**A**) ELISA to show the dose-dependent binding of sCD163-FITC-Hb complex to IgG immobilized on MaxiSorp plates. FITC-BSA was used as negative control in place of FITC-Hb. All the readings were subtracted from the values obtained with addition of Hb-FITC alone. *$p < 0.05$. (**B** and **C**) Representative sensograms of three independent surface plasmon resonance experiments showing the dose-dependent binding profiles between immobilized IgG to (B) Hb (0.2–0.8 μM) and (C) Hb (0.2 μM) plus sCD163 (2.5–10 ng/ml). Response units (RU) for (B) were dual referenced against BSA-*N*-acetylglucosamine–immobilized reference flow cell and BSA (0.2–0.8 μM) whereas (C) was referenced against sCD163 only (without Hb) controls. Dashed lines represent the curve fitting. (**D**) Wild-type, CD64-, CD32-, and CD16-silenced primary monocytes were incubated with the sCD163-Hb-IgG complex (0.5–2 μM) for 30 min and endocytosis was quantitated using the CyAn ADP flow cytometer on the FITC channel. Data are representative of three independent experiments using primary monocytes from a single healthy donor.

sCD163-Hb-IgG complex was recycled to the membrane. Thus far, our results corroborate that mCD163 plays a major role in frontline defense as it binds Hb to reduce the POX activity, whereas the shedded sCD163 further scavenges plasma Hb, re-enters the monocyte, and undergoes recycling into mCD163, thus completing the autocrine cycle of Hb detoxification and CD163 renewal.

Because the endocytosed sCD163 is recycled to the cell surface, we queried the fate of the internalized Hb. HO-1 is an enzyme responsible for the catabolism of heme into biliverdin, carbon monoxide, and iron (45). Results showed that Hb induced a 70% increase in the HO-1 activity relative to negative control (Fig. 5C). Importantly, activated Hb induced 30% higher HO-1 activity compared with native Hb. Conceivably, this timely induction

Table I. siRNA pool used to knock-down human FCGRI, FCGRII, and FCGRIII

| Gene | siRNA Sequence (5′–3′) |
|---|---|
| FCGRI (CD64) | AAACAAAGUUGCUCUUGCA |
| | GGAAAUGUCCUUAAGCGCA |
| | GGAACACAUCCUCUGAAUA |
| | GAGAAGACUCUGGGUUAUA |
| FCGRII (CD32) | rArGrArArCrArArArGrArGrCrCrCrArArAUrUrArCrCrArGAA |
| | rGrArUrGrUrArGrCrArArCrArUrGrArGrArArArCrGrCrUTA |
| | rGrArArUrUrArGrArGrArGrGrUrGrArGrGrArUrCrUrGrGTA |
| FCGRIII (CD16) | rGrCrUrUrCrGrCrUrGrArGrUrUrArArGrUrUrArUrGrArAAC |
| | rCrGrArUrGrArGrGrUrCrCrUrCrUrUrArArUrGrCrUrArGrGAG |
| | rArGrArArArUrArGrCrCrArGrGrUrArGrUrCrCrArGrGrArUAG |
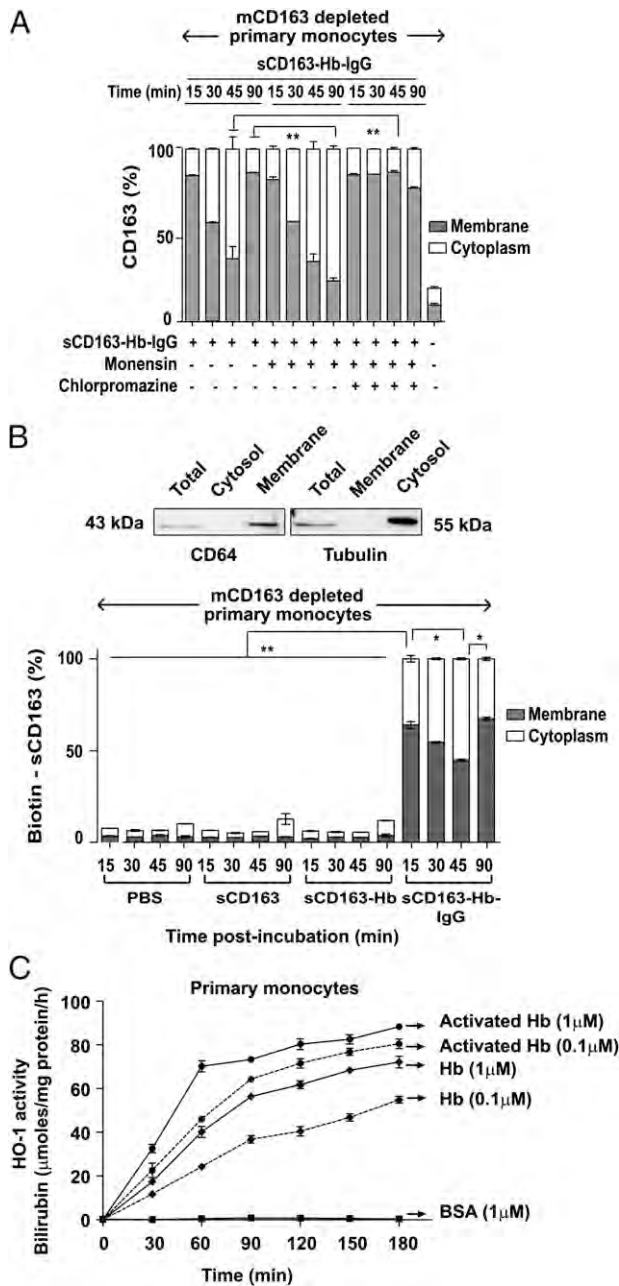
**FIGURE 5.** The endocytosed sCD163 is recycled into mCD163 whereas the internalized Hb is catabolized. (**A**) Primary monocytes predepleted of mCD163 and treated with 5 μg/ml CHX were incubated with 1.5 μM sCD163-Hb-IgG complex for up to 90 min with or without pretreatment with 70 μM chlorpromazine (inhibitor of endocytosis) and 20 μM monensin (inhibitor of early endosome recycling) for 60 min. CD163 was quantified in the membrane and cytosol fractions using sandwich ELISA. (**B**) *Top panel*, Purity of membrane/cytosol fractions was tested using membrane (CD64) or cytosolic (tubulin) markers. *Bottom panel*, mCD163-depleted primary monocytes were incubated with either biotinylated sCD163 alone or biotin sCD163-Hb-IgG complex for up to 90 min. Biotinylated sCD163 was quantified in membrane or cytosolic fractions using streptavidin-HRP by ELISA. (**C**) Primary monocytes were stimulated with increasing doses of Hb (0.1, 1 μM) for up to 180 min, and HO-1 activity (μmoles bilirubin/mg protein/h) was measured by spectrometric quantitation of bilirubin in the presence of excess substrate. Data are representative of three independent experiments using primary monocytes from a single healthy donor. $*p < 0.05$, $**p < 0.005$.

of HO-1 activity detoxifies the internalized Hb and preempts the avalanche of superoxide radicals resulting from the endocytosed redox-active Hb.

*Hb induces cell–cell communication between monocytes and endothelial cells via sCD163 and IgG*

Next, we queried the cellular physiological significance of the monocyte-derived sCD163. Because monocytes are in contact with endothelial cells in vivo, it is conceivable that sCD163 acts in a paracrine fashion to communicate /alert the proximal cells of the imminent presence of cytotoxic Hb. To test this, we used primary HMVEC, which are known to endogenously express FcγRII (CD32) (26) but lack CD163 (46). We then measured the induction of HO-1 in HMVEC incubated for 6 h with increasing doses of the sCD163-Hb-IgG complex. Results showed that when compared with just Hb alone or other negative controls, the sCD163-Hb-IgG complex upregulated HO-1 levels by 3-fold (Fig. 6A). The induction of HO-1 was dose-dependent of the sCD163-Hb-IgG complex (Fig. 6A, box). This indicates that sCD163 and IgG mediate the Hb-induced transactivation of the endothelial cells. After internalization into HMVEC, the Hb is catabolized by HO-1.

To assess the potential Hb-induced crosstalk between the monocytes and endothelial cells, we cocultured the two cell types in the presence of Hb and measured the cytokine production by the cells. To confirm the significance of CD163 in this process, we employed CD163$^+$ primary monocytes or CD163$^-$ THP-1 monocytes (control) (47). Fig. 6B (box) shows a synergistic increase in the production of TNF-α, IL-8, and IL-10 when the HMVEC were cocultured with CD163$^+$ primary monocytes compared with THP-1 or when stimulated in isolation. This synergy was lost when the monocytes were preincubated with anti-CD163, suggesting that the monocyte-derived sCD163 is indispensable for the activation of endothelial cells, which lacks endogenous CD163. Furthermore, stimulation of HMVEC with the sCD163-Hb-IgG complex elicited higher amounts of TNF-α and IL-8 compared with individual protein controls (Supplemental Fig. 4A), corroborating our coculture results (Fig. 6B). Thus, the monocyte-derived sCD163 mediates the paracrine activation of the proximal endothelial cells to systemically alert the human body on the imminent toxicity of plasma Hb.

Next, we tracked CD163 and Hb in the HMVEC, which had been cocultured with primary monocytes or THP-1 monocytes for 45 min. Fig. 6C shows that both CD163 and Hb are colocalized in HMVEC only when cocultured with CD163$^+$ primary monocytes but not with CD163$^-$ THP-1 cells. Consistently, the colocalization of Hb and CD163 within the HMVEC was observed only when the two proteins were presented as a complex of sCD163-Hb-IgG (Supplemental Fig. 4B). Additionally, in the absence of IgG, no endocytosis of Hb was detected in HMVEC even when cocultured with CD163$^+$ monocytes, suggesting that IgG is required to bridge the sCD163-Hb complex to FcγR on the HMVEC. Also, when HMVEC and CD163$^+$ monocytes were cocultured in the absence of Hb, no CD163 entered the HMVEC. By live cell imaging, we have demonstrated the sCD163-mediated interaction between monocytes and the proximal endothelial cells in the presence of Hb and IgG (Supplemental Videos 8–11).

Altogether, we have shown that sCD163 is recycled to achieve homeostasis of mCD163 on the monocytes and, simultaneously, the sCD163-Hb complex induces the monocytes to collaborate with the proximal endothelial cells via IgG-FcγR. Whereas the dynamic importation of plasma Hb-sCD163 shuttles the cytotoxic cargo of Hb into the monocytes in an autocrine cycle, it also transactivates the endothelial cells in a paracrine manner to secrete cytokines to raise a systemic alert on the imminent danger from the redox-active Hb (Fig. 7). The internalized Hb is catabolized by HO-1 in both monocytes and endothelial cells.
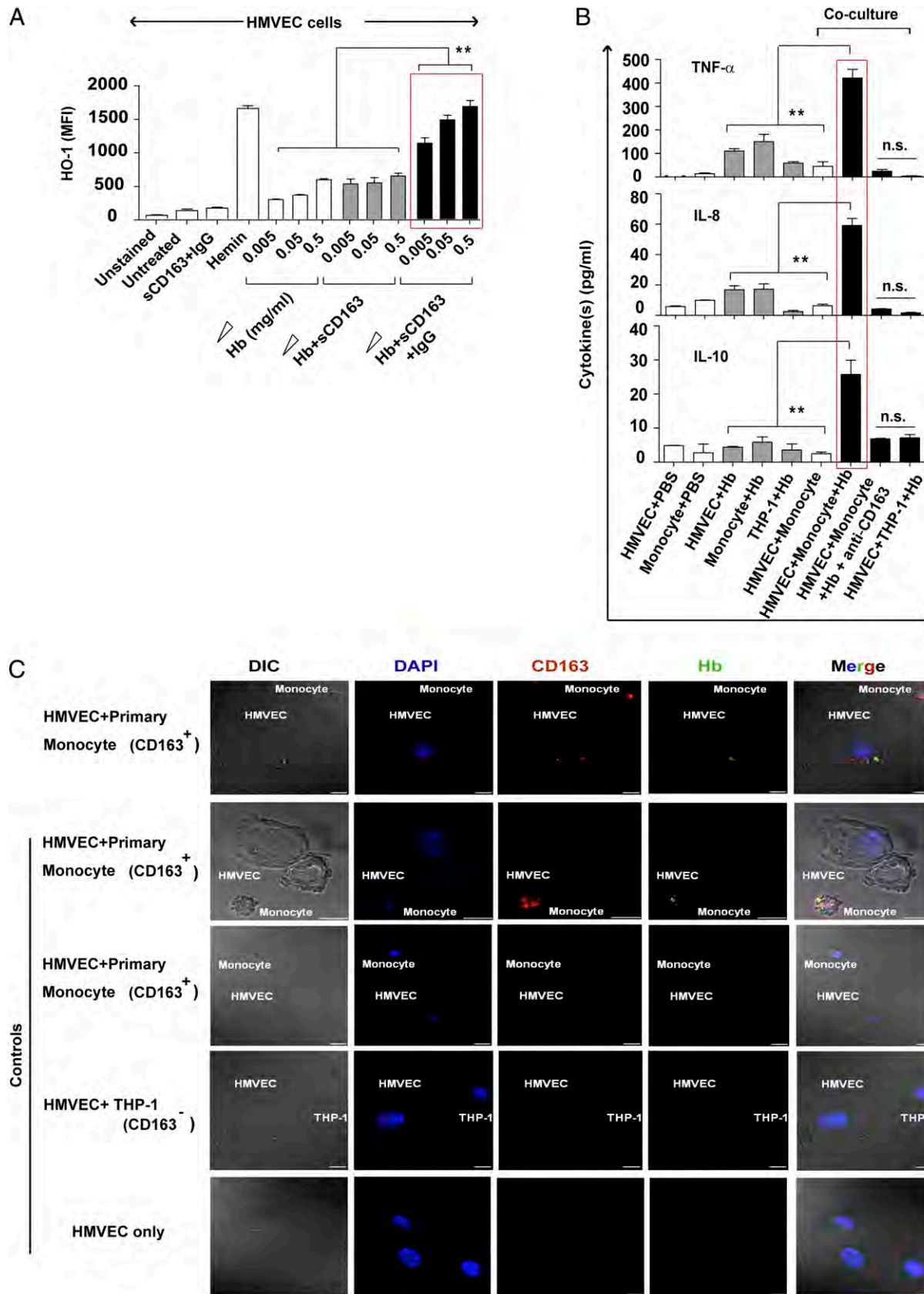
**FIGURE 6.** Hb induces cell–cell communication between monocytes and endothelial cells via sCD163 and IgG. (**A**) HMVEC were stimulated with increasing doses of Hb alone or preformed sCD163-Hb-IgG complex for 6 h, and HO-1 protein was quantified by FACS. Hemin was used as positive control. (**B**) Cytokine production when HMVEC were cocultured for 24 h with CD163^{+} primary monocytes or CD163^{−} THP-1 cells in the presence of 0.5 mg/ml Hb. (**C**) Immunostaining to track localization of sCD163 and Hb in HMVEC cocultured with either CD163^{+} primary monocytes or CD163^{−} THP-1 cells in the presence of Hb for 45 min. All images were obtained using the LSM 510 META confocal microscope under ×100 oil objective. Scale bars, 10 μm. Data represent the means ± SEM of three independent experiments with primary monocytes from single donor. **$p < 0.005$.

**FIGURE 7.** A hypothetical model of Hp-independent intravascular detoxification and clearance of cell-free Hb by CD163. Hemolysis ruptures RBCs and releases cytotoxic Hb into the plasma. Upon recruitin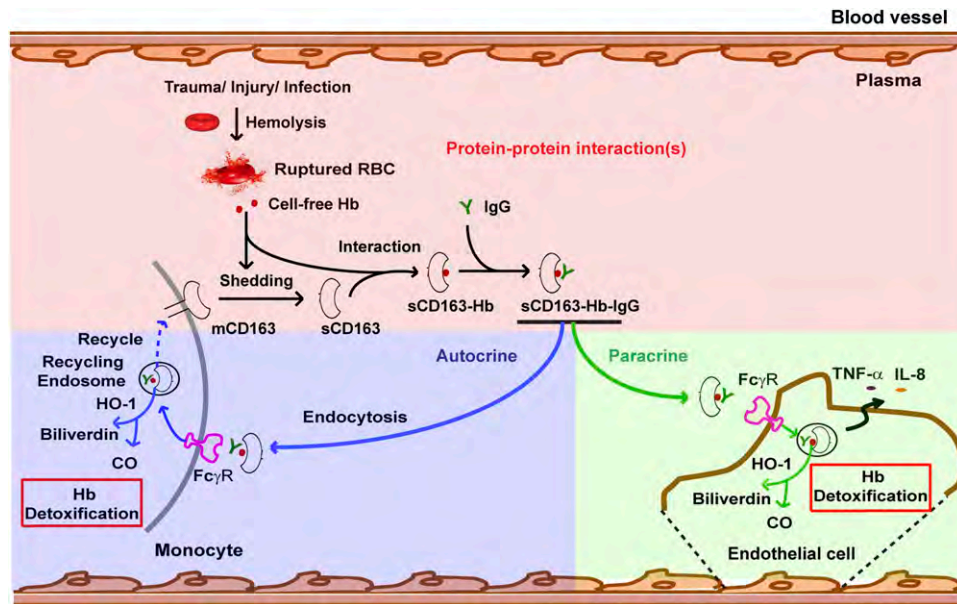g Hb, the mCD163 directly suppresses the POX activity of Hb in situ on the monocyte membrane. Hb induces shedding of mCD163 into the plasma, and the resulting sCD163 further captures and quenches the residual redox-reactive Hb. Subsequently, IgG interacts with the sCD163-Hb complex. The sCD163-Hb-IgG complex then 1) elicits an autocrine loop of endocytosis via FcγR on the monocyte and subsequent recycling of the internalized sCD163 via endosomes to restore mCD163 homeostasis, whereas the internalized Hb is catabolized by HO-1; and 2) induces the paracrine transactivation of the neighboring endothelial cells (represented by HMVEC tested in this study) lining the blood vessel causing them to upregulate HO-1 and secrete cytokines to mount a systemic defense against Hb.

## Discussion

Redox-active extracellular Hb results in oxidative stress and cytotoxicity (36). Hence, it is crucial for our blood cells to counteract the pro-oxidative Hb at the immediate outset even before its uptake into the cells. Although, it is known that mCD163 directly interacts with Hb independent of Hp (18), the functional impact of this interaction on Hb redox reactivity remains enigmatic. We have discovered and mapped in detail a novel two-pass detoxification mechanism of Hb by CD163, independent of Hp. First, at the outset of the encounter with plasma Hb, mCD163 directly inhibits the Hb POX activity in situ and rescues monocytes from Hb-triggerred apoptosis (Fig. 1, Supplemental Fig. 1). The mCD163 is also shed into the plasma (Fig. 2). The resulting sCD163 scavenges residual free Hb and upon endocytosis of the sCD163-Hb complex via IgG-FcγR, the sCD163 is recycled to restore homeostasis of mCD163 in an autocrine cycle, whereas the internalized Hb is catabolized by HO-1. Second, this novel mechanism of clearance of Hb by CD163 transactivates the proximal endothelial cells in a paracrine fashion, causing these cells to upregulate HO-1 and inducing secretion of cytokines, thus mounting a systemic immune defense against Hb.

Besides suppressing Hb POX activity at the monocyte surface, CD163, which is cotranslocated into the cells, also downregulates the generation of intracellular ROS from the endocytosed Hb (Fig. 1C, Supplemental Videos 1–7). In the absence of such a mechanism as illustrated in this study with CD163⁻ cells, the hydrophobic nature of the Hb heme could readily permeate the cells, inducing free radicals, which would lead to lipid peroxidation and cell death (48, 49). Having established the direct inhibition of the redox activity of Hb by mCD163 (independent of Hp), we then queried the pathophysiological significance of sCD163 under severe hemolytic condition. We found that sCD163 binds excess plasma Hb dose-dependently and rapidly downregulates the Hb POX activity (Fig. 3A, 3B). Thus, it is conceivable that during a severe hemolysis, such a "capture and quench" action by sCD163 would constitute an ef-

fective host defense strategy to sequester the heme iron and pre-empt its redox activity. Of particular importance is that the resulting sCD163-Hb complex, which is still redox-active, must be rapidly and efficiently removed from circulation so as to subvert the Hb iron–mediated cytotoxicity. To this end, we identified IgG as a novel interaction partner participating with the sCD163-Hb complex to enable endocytosis of the sCD163-Hb-IgG complex via FcγR into the monocytes (Fig. 4). We found that interaction of the sCD163-Hb complex with IgG is a critical prerequisite for subsequent endocytosis of the complex into monocytes via FcγR (Supplemental Fig. 3).

Following endocytosis of the sCD163-Hb-IgG complex, the internalized sCD163 is recycled via early endosomes to the cell membrane to restore mCD163 (Fig. 5). This is also supported by reports documenting that many endocytic receptors are recycled when internalized into the cell (50) and that early endosomes serve as the focal points of the endocytic pathway, enabling them to undergo fast recycling to the plasma membrane (51). During a severe hemolysis, such a dynamic and efficient recycling of sCD163 would presumably potentiate the recovery of mCD163, which acts to fortify the monocytes against the cytotoxic avalanche of free radicals generated by the cell-free Hb POX. Furthermore, using coculture experiments, we established that sCD163, in collaboration with IgG, confers Hb-scavenging ability to the proximal endothelial cells and also transactivates them to respond against the Hb (Fig. 6, Supplemental Fig. 4). Such a crosstalk between monocytes and endothelial cells (mediated by sCD163-Hb-IgG complex via FcγR) mounts a systemic defense against toxic Hb. Overall, CD163 is dynamically deployed in a two-pass detoxification tactic to engage with and suppress the pro-oxidant activity of plasma Hb, whereas its residential level on the monocyte membrane is restored to homeostasis in an efficient autocrine cycle. Simultaneously, it also transactivates adjacent endothelial cells in a paracrine fashion to metabolize the endocytosed Hb and secrete cytokines to systemically alert the imminent presence of a danger molecule, Hb (Fig. 7).

## Acknowledgments

## Disclosures

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<u>**Poster #3019**</u>

### Regulation of hemoglobin toxicity and inflammation by scavenger receptor mediated monocyte-endothelial crosstalk

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High concentrations of extracellular hemoglobin (Hb) accumulate intravascularly and in tissues under clinical settings such as injury, inflammation, trauma or infection. Cell-free Hb is highly redox-active and generates cytotoxic reactive oxygen species, leading to the synergistic induction of inflammation along with other damage- and pathogen- associated molecular patterns (DAMPs and PAMPs, respectively) during hemolysis and sepsis. The large amounts of plasma hemoglobin quickly saturate haptoglobin (Hp), the primary anti-oxidant defense in the blood. Hence, survival during severe hemolysis depends on rapid clearance of the pro-inflammatory Hb from circulation. Here, we identified a novel mechanism by which the monocyte scavenger receptor, CD163 and plasma IgG collaboratively induce monocyte-endothelial crosstalk to detoxify and clear Hb, independent of Hp. Using time lapse confocal microscopy and biochemical interaction assays, we showed that both membrane bound receptor and soluble forms of CD163, shedded into the plasma upon stimulation with Hb dynamically collaborate with each other to 'capture and quench' the redox-active Hb *in-situ*. Interestingly, we identified plasma IgG to be a part of the Hb-CD163 interactome, playing a crucial role in trafficking the complex into the monocytes for detoxification and clearance. Using *ex-vivo* co-culture experiments and real-time imaging, we found that monocyte-derived soluble CD163 transactivated the proximal endothelial cells to secrete cytokines and mount a systemic defense against the inflammatory Hb.