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

*J Immunol* 2013; 190:5267-5278; Prepublished online 15 April 2013;
doi: 10.4049/jimmunol.1202648
http://www.jimmunol.org/content/190/10/5267

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/04/15/jimmunol.1202648.DC1

**References**
This article cites 50 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/190/10/5267.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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* ‡

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.

H emolysis 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 as superoxide anion \((\text{O}_2^-)\), 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 FcγRII (CD64) and goat polyclonal anti-human CD163 were purchased from R&D Systems. Purified mouse anti-human FcγRII (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% CO2 at 37°C in HEPES-buffered RPMI 1640 (Invitrogen) containing 100 μM penicillin, 100 μg/ml streptomycin, and 10% FBS. The cell density of 2 × 10^6 cells/ml. HEK293T and HepG2 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 100 μM 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 O2·− production

The generation of free radicals (O2·−) 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 O2·−.

Extraction of native membrane proteins and cytosolic proteins

The native membrane and cytosolic proteins from 2 × 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 on ice until thawed. The cell suspension was then washed twice with 2 ml ice-cold extraction buffer I supplemented with protease inhibitor mixture. The insoluble material was pelleted by centrifugation at 16,000 × 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 × 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 × 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-H2DCFDA dye

The ROS generated within the monocytes was measured using the cell permeant oxidation-dependent fluorescent dye CM-H2DCFDA (Invitrogen). SU-DHL-1 cells were plated at 2 × 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-H2DCFDA 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 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 × 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 × 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-goat; R&D Systems). Then the cells were washed three times with PBS, and 10^5 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 KIPA lysis buffer (Cell Signaling Technology) containing 1 mM PMFS 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 × 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 × 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 × 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 with 4% (v/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 × 10^6 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 (Amersham). 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 crosslinked 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 ethanalamine 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 (R&D Systems) 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 CMS 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-acytelylglucosamine–immobilized reference flow cells (negative control).

Silencing of FcγR in primary monocytes
To validate the role of FcγR in the uptake of IgG-Hb-sCD163, 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 × 10^6) were nucleofected with 2 μg siRNA pool using the Amaxa Nucleofector (human monocyte Nucleofector kit, Nucleofector program Y-01). 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-biotin; Pierce) was used for biotinylation of sCD163. Briefly, 2 μg/ml sCD163 was incubated with 20-fold molar excess of sulfo-NHS-S-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 biotin-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 μM 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 (ε = 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) 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 pre-incubation with anti-CD163. Fig. 1C (left panel) shows that activated Hb induced ∼75% higher ROS production than did native...
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 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. Reciprocally 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...
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.

FcyR 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 FcyR 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 FcyR, which might participate in the sCD163-Hb interaction. We found that indeed Hb in the sCD163-Hb complex communoprecipitated 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 (KD = 1.15 × 10−7 M) and between IgG, Hb, and sCD163 (KD = 2.25 × 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 FcyR 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 FcyR knockdown cells. The efficiency of knockdown of all three types of FcyRs, that is, FcyRI (CD64), FcyRII (CD32), and FcyRIII (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 FcyR 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 FcyR 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 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 FcyR on the monocyte.

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-HP. 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
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

<table>
<thead>
<tr>
<th>Gene</th>
<th>siRNA Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCGRI (CD64)</td>
<td>AAAACAAAGUUGCUCUUGCA</td>
</tr>
<tr>
<td></td>
<td>GGAAAUGUCCUUAAGCGCA</td>
</tr>
<tr>
<td></td>
<td>GGAACACAUCCUCUGAAUA</td>
</tr>
<tr>
<td></td>
<td>GAGAAGACUCUGGGUUAUA</td>
</tr>
<tr>
<td>FCGRII (CD32)</td>
<td>rArGrArArArCrArCrCrCrArArUrArCrArCrArGAA</td>
</tr>
<tr>
<td></td>
<td>rGrArUrGrUrArGrCrArArUrGrArGrArArCrGrCrUTA</td>
</tr>
<tr>
<td></td>
<td>rGrArUrUrArGrGrArGrUrGrGrArGrUrCrUrGrGTA</td>
</tr>
<tr>
<td>FCGRIII (CD16)</td>
<td>rGrCrUrUrCrGrCrUrGrUrArGrUrArGrUrArGrUrArAAC</td>
</tr>
<tr>
<td></td>
<td>yCrGrArUrGrUrCrCrUrCrUrArUrArGrUrArGrGAG</td>
</tr>
<tr>
<td></td>
<td>rArGrArArArUrArCrGrGrGrUrGrUrCsCrArGrAeUAG</td>
</tr>
</tbody>
</table>
membrane and cytosol fractions using sandwich ELISA. 

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 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 catalyzed 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+ primary monocytes, suggesting that IgG is required to bridge the sCD163-Hb complex to FcγRII 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-FcγRI. 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 catalyzed by HO-1 in both monocytes and endothelial cells.

**FIGURE 5.** The endocytosed sCD163 is recycled into mCD163 whereas the internalized Hb is catalyzed. (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/cytosolic 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 (μmol 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.
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 cytoxic Hb into the plasma. Upon recruiting 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-triggered apoptosis (Fig. 1, Supplemental Fig. 1). The mCD163 is also shedded 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 co-translocated 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^-^^-^-^-^-^-^-^-^-^-^-^-^^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^
Acknowledgments
We thank Tong Yang, National University of Singapore Centre for BioImag- ing Sciences, for technical assistance with the confocal microscopy.

Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Data**

**CD163 and IgG Co-Defend Against Cytotoxic Hemoglobin via Autocrine and Paracrine mechanisms**

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

**Supplemental Figure legends S1-S4**

**Figure S1. CD163 detoxifies Hb and rescues cells from Hb-induced apoptosis.**

(A) 100 μg Hb was incubated with subtilisin A (0-9 U) for 30 min at 37°C (left panel) and subtilisin A (1.5 U) for a time course (right panel). 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 is indicated by the bands below 10 kDa (red box), which is coincident with the accompanying rise in POX activity. Incubation with 1.5 U of subtilisin A for 15 min was henceforth used consistently to generate activated Hb. (B) The pseudoperoxidase (POX) activity of 10 μg native Hb incubated 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 isoform) was used as a positive control. Red box indicates progressive decrease in Hb-POX activity with increasing doses of mCD163. (C) Intracellular ROS and cell viability of HEK293T and HepG2 cells stimulated with 100 μg cell-free Hb. * indicates p < 0.05; n.s., not significant. (D) Real-time production of Hb-generated intracellular ROS in primary monocytes in the presence or absence of functional CD163, quantified as the mean fluorescence intensity of CM-H2DCFDA. (E) Dynamics of apoptosis in cells stimulated with 15 μM of native Hb or activated Hb with or without pretreatment with 0.1 μg/ml anti-CD163 using the Annexin-V FITC and propidium iodide staining. The percentage of late-apoptotic cells (red box) in the two-parameter histogram plot is indicated.

**Figure S2. sCD163 captures Hb and restores mCD163 level.**

(A) Co-immunoprecipitation to test for interaction between Hb and sCD163 immobilized using Protein A Sepharose beads. Positive controls included the purified proteins, sCD163 and Hb, to verify the molecular mass. (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. (C) Flow cytometry to study the dose-dependent effect of activated Hb (0.1, 1 μM) on mCD163 in the presence or absence of 10 ng/ml sCD163.

**Figure S3. Hb in the sCD163-Hb complex interacts with IgG.**

(A) Immunostaining and FACS analysis of CD163 and Hb in primary monocytes pre-depleted of CD163 by treatment with PMA and then cycloheximide (CHX) treatment followed by incubation with either sCD163 alone or sCD163-FITC Hb, in absence of serum. Scale bars, 5 μm. (B) Top panel- co-immunoprecipitation to test for interaction
between IgG and Hb. Negative control included Hb incubated with beads alone without IgG. Bottom panel- co-immunoprecipitation to test for interaction between IgG, Hb and sCD163. BSA replaced Hb in the negative control. (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. (D) FACS to analyze the efficiency of CD64, CD32 and CD16 knockdown in primary monocytes 48h post nucleofection. Scrambled siRNA-nucleofected primary monocytes were used as negative control.

Figure S4. sCD163, in collaboration with IgG, transactivates endothelial cells to respond to Hb.

(A) Cytokine production by HMVEC cells stimulated for 24 h with increasing doses of Hb alone or pre-formed sCD163-Hb-IgG complex. (B) Immunostaining to track sCD163 and Hb in HMVEC cells incubated with pre-formed sCD163-Hb-IgG complex for 30 min. Images were acquired using LSM510 confocal microscope under the 100x oil objective. Scale bars, 10 μm. ** indicates p< 0.005; n.s., not significant.

Movies S1-S7: Real-time imaging of intracellular ROS production in cells induced by cell-free Hb

**Movie S1.** Primary monocytes loaded with 5 μM CM-H2DCFDA, were stimulated with 1.5 μM native Hb. The ROS-fluorescence (green) in the cells gradually increased to peak at 50 s and subsequently decreased steadily to near background levels by 200 s.

**Movie S2.** Primary monocytes loaded with 5 μM CM-H2DCFDA, were stimulated with 1.5 μM activated Hb. The ROS-fluorescence (green) in the cells was substantially increased over 100 s and then gradually subsided by 200 s.

**Movie S3.** Primary monocytes pre-treated with 0.1 μg/ml anti-CD163 were loaded with 5 μM CM-H2DCFDA and then stimulated with activated 1.5 μM Hb. The CD163-mediated reduction in the ROS-fluorescence (green) in the cells was abrogated by the action of anti-CD163.

**Movie S4.** Primary monocytes were loaded with 5 μM CM-H2DCFDA and stimulated with 1.5 μM activated Hb in the presence of a ROS scavenger, N-acetyl cysteine (1 mM), which completely scavenged the ROS generated.

**Movie S5.** Untreated primary monocytes were loaded with 5 μM CM-H2DCFDA. Only background levels of ROS-fluorescence (green) were observed in cells.

**Movie S6.** Primary monocytes loaded with 5 μM CM-H2DCFDA and stimulated with 1.5 μM human serum albumin (HSA) as a negative control. Only background levels of ROS-fluorescence (green) were observed in the cells indicating the specificity of Hb-POX driven ROS production.

**Movie S7.** Intracellular ROS production persisted in Jurkat cells. Jurkat cells, which do not harbor endogenous CD163, were loaded with 5 μM CM-H2DCFDA and stimulated
with 1.5 μM activated Hb. The ROS generated in the cells was intense and the green fluorescence steadily increased with time, plausibly due to the entry and accumulation of the hydrophobic Hb-heme.

**Movies S8-S11: Live cell imaging of Hb induced monocyte-endothelial interactions ex-vivo**

**Movie S8.** Cell-cell interaction of co-cultured primary monocytes and endothelial cells stimulated with 1μg Hb in the presence of IgG. Functional sCD163 and IgG are crucial to mediate the Hb-induced interaction between monocytes and endothelial cells. EC-HMVEC M- Monocyte

**Movie S9.** Co-cultured primary monocytes and endothelial cells stimulated with 1μg Hb in the absence of IgG. No interaction was observed in the absence of IgG suggesting that IgG is crucial for the sCD163-mediated monocyte-endothelial cross talk. EC-HMVEC M- Monocyte

**Movie S10.** Co-cultured primary monocytes and endothelial cells not stimulated with Hb, but in the presence of IgG. IgG by itself in the absence of Hb did not induce any interaction between monocytes and endothelial cells. EC-HMVEC M- Monocyte

**Movie S11.** Endothelial cells were co-cultured with primary monocytes pre-incubated with anti-CD163 and stimulated with Hb in the presence of IgG. Immunodepletion of CD163 abolished the monocyte-endothelial interaction suggesting that CD163 is indispensible for the interaction. EC-HMVEC M- Monocyte
Figure S1

**A**

<table>
<thead>
<tr>
<th>Subtilisin A (U)</th>
<th>Native Hb (100 μg)</th>
<th>Subtilisin A (1.5 U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>SU-DHL-1</th>
<th>HEK293T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>HEK293T</th>
<th>HepG2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th>Intracellular ROS</th>
<th>Mean fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**E**

<table>
<thead>
<tr>
<th>Propidium Iodide</th>
<th>FITC-Annexin V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure Legend:**

- **SU-DHL-1** and **HEK293T** cell lines are used for the experiments.
- **Native Hb** and **Activated Hb** are the two treatments.
- **NAC+Activated Hb** is used as a control.
- **Hb+NAC** and **HSA** are also included in the experiments.
- **FITC-Annexin V** and **Propidium Iodide** are used for cell viability analysis.
- **Jurkat+Hb** and **anti-CD163+Hb** are used as additional controls.
- **Time (min)** and **Time (s)** are used to measure the duration of the experiments.
- **Mean fluorescence intensity** and **Fluorescence Intensity (AU)** are used to measure the intensity of the fluorescence.
Figure S2

A

Beads + sCD163 + Hb
Flow-through

16 kDa
130 kDa

Hb
sCD163

16 kDa
130 kDa

Positive control
Non-treated
Treated

Activated Hb (0.1 μM)
Activated Hb (1 μM)

Activated Hb (0.1 μM)
Activated Hb (1 μM)

- sCD163
+ sCD163

10 ng/ml
5 ng/ml
0 ng/ml

mCD163 (% relative to untreated)

FITC log

Events

Time (h)

0
1
2
3
Figure S4

A

B

DIC DAPI Hb sCD163 Merge

Untreated

20 Ab only

Hb only

Hb+sCD163

Hb+IgG

sCD163+IgG

Hb+sCD163+IgG