Preferential Silent Survival of Intracellular Bacteria in Hemoglobin-Primed Macrophages

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Abstract

Hemolysis releases hemoglobin (Hb), a prooxidant, into circulation. While the heme iron is a nutrient for the invading pathogens, it releases ROS, which is both microbicidal and cytotoxic, making it a double-edged sword. Previously, we found a two-pass detoxification mechanism involving the endocytosis of Hb into monocytes in collaboration with vascular endothelial cells to overcome oxidative damage. This prompted us to examine the effect of Hb priming on host cell viability and intracellular bacterial clearance during a hemolytic infection. Here, we demonstrate that Hb-primed macrophages harbor a higher intracellular bacterial load but with suppressed apoptosis. p-ERK and p-p38 MAPK were significantly downregulated, with concomitant impairment of Bax and downstream caspases. The Hb-primed cells harboring intracellular bacteria upregulated anti-inflammatory IL-10 and downregulated proinflammatory TNF- α , which further enhanced the infectivity of the neighboring cells. Our findings suggest that opportunistic intracellular pathogens ex-

ploit the Hb-scavenging machinery of the host to silently persist within the circulating phagocytes by suppressing apoptosis while escaping immune surveillance.

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Introduction

Hemolysis caused by infection [1], injury, trauma [2], or blood transfusion [3] releases the prooxidant hemoglobin (Hb) into the plasma, which generates damaging reactive oxygen species (ROS) [4]. During severe hemolysis, the redox-active cytotoxic Hb reaches excessively high concentrations (e.g. 0.6 mg/ml in sickle cell anemia [5] and 0.5–2 mg/ml in paroxysmal nocturnal hemoglobinuria [6]) in the plasma, which can be life threatening [7]. Plasma proteins such as haptoglobin (Hp) [8] and alpha-1-microglobulin [9] bind to free Hb or heme in the plasma and confer protection against the cytotoxic Hb. In self-defense against the prooxidative Hb, phagocytes such as monocytes and macrophages express a variety of scavenger receptors such as CD163 which recognize the Hb-Hp complex [10] and become effectively loaded with Hb. We recently showed that under severe hemolytic

conditions CD163 collaborates with IgG and Fc receptors to endocytose and detoxify plasma Hb in an Hp-independent manner and induces monocyte-endothelial cross talk [11]. Therefore, during an infection by hemolytic pathogens, the phagocytes could become effectively primed with Hb along with the invading pathogen. Opportunistic pathogens such as *Staphylococcus aureus* have been reported to invade and survive intracellularly within phagocytes during infection [12, 13] and are known to utilize the heme iron for survival [14]. Consistently, patients with hemolytic disorders such as malaria and paroxysmal nocturnal hemoglobinuria [15] are predisposed to infections [16] especially by intracellular bacteria [17]. Other studies in mice have also shown that hemolysis increases the susceptibility to bacterial infections [18]. This prompted us to hypothesize that uptake of Hb during episodes of hemolysis might predispose the macrophages to intracellular infection by opportunistic bacteria.

Most mechanisms that have been proposed to account for the susceptibility of hemolytic patients to intracellular bacterial infections involve dysfunctions of monocytes and macrophages. Hemolysis may impair the functions of monocytes and macrophages by direct adhesion of infected RBCs [19], through the accumulation of hemozoin within these cells [20], or via the impairment of systemic IL-12 production [21]. Another recent study showed that resistance to intracellular bacterial infection is impaired as a result of neutrophil dysfunction caused by the liberation of heme during hemolysis [22]. Hence, although there is compelling evidence to suggest that hemolysis results in susceptibility to infections, the underlying molecular mechanism remains unclear.

Here, we aimed to investigate the effect of Hb priming of macrophages on the fate of the invading bacteria as well as host cell survival. We used *S. aureus* and *Salmonella typhimurium* as representative intracellular bacteria because they have been reported to survive intracellularly in phagocytes [23, 24]. As a control, we used *Pseudomonas aeruginosa*, which has been reported to reside extracellularly during infection [25]. We found that Hb-primed macrophages harbored a higher bacterial load upon infection with intracellular bacteria such as *S. aureus* and *S. typhimurium* and apoptosis was apparently suppressed in these infected macrophages. In contrast, we observed that infection of the Hb-primed macrophages with extracellular-resident bacteria (*P. aeruginosa*) induced cell death. Our results suggest that the intracellular bacteria might be exploiting the Hb-scavenging mechanism of the host to their advantage by

hijacking the heme iron for their nutritional benefit while simultaneously inhibiting host cell apoptosis for their survival. We propose a molecular mechanism to explain how Hb-primed macrophages may constitute a silent survival niche for the invading microbes.

Materials and Methods

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

Hb and Pyrogen-Free Buffers

Human Hb metHb (Hb-Fe³⁺; Sigma) was purified from adult Hb. It consisted of 96.5–98.5% HbA1 and 1.5–3.5% HbA2 (chemical data sheet, Sigma catalog No. H7379). The same batch of Hb was consistently used for all of the experiments described. The purified Hb was tested for potential endotoxin contamination using a PyroGene assay (Lonza Inc.). The endotoxin level was found to be ≤ 0.05 endotoxin units/ml, an acceptable low limit of endotoxin. To minimize endotoxin contamination, all of the glassware was depyrogenated via baking at 200°C for 2 h. All of the buffers and media were prepared using pyrogen-free water (Baxter Healthcare) and handled under sterile conditions.

Human Primary Cells and Cell Cultures

U937 cells (human leukemic monocyte lymphoma cells) were cultured in RPMI 1640 containing 10% (v/v) FBS at 37°C in an atmosphere of 5% CO₂. Cell cultures were maintained at an exponential growth rate via media replacement every 2–3 days and cells were provided fresh media the day before they were used for experiments. The U937 cells were differentiated into macrophages by stimulation with 1×10^{-8} M phorbol myristic acid (PMA) and 2.5×10^{-7} M dexamethasone for 3 days in complete medium. The reason for using dexamethasone-treated U937 cells was to upregulate the expression of the Hb receptor CD163 and differentiate them into macrophages which would uptake Hb [26] (see online suppl. fig. S1). Dexamethasone-differentiated monocytes have been reported to serve as a model system to study Hb uptake, and glucocorticoid treatment in vitro and in vivo has been found to shift monocyte differentiation towards a phenotype with a high Hb clearance and detoxification capacity [27]. To prove that the observed phenotypes were indeed due to Hb uptake and not dexamethasone treatment, we included an experimental control in which the cells were treated with 1×10^{-8} M PMA and 2.5×10^{-7} M dexamethasone but were not treated with Hb (denoted Unt for untreated).

Primary monocytes were obtained by first isolating peripheral blood mononuclear cells from human blood obtained from healthy individuals using Ficoll-Paque premium density gradient media (GE Healthcare). The primary monocytes were then isolated by negative selection using an EasySep Human Monocyte Enrichment Kit (STEMCELL). The primary monocytes were seeded overnight at 0.8×10^6 cells/well in 24-well plates and maintained in complete RPMI 1640 at 37°C and 5% CO₂.

Bacterial Strains and Storage

The laboratory strains *S. aureus* PC1839 (kanamycin-resistant, V8 protease+), *S. enterica* serovar Typhimurium strain LT2, and *P. aeruginosa* strain PAO1 were used in this study. The cultures were stored 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 $\mu\text{g}/\text{ml}$)-supplemented LB was used as the selective growth medium for *S. aureus*.

Protease Activity Assay

S. aureus and *S. typhimurium* were cultured overnight at 37°C in the protease-producing buffer. Secondary cultures were grown to an exponential phase and the protease activity in the culture supernatant was determined at 37°C using a 1% azocasein assay [28]. One unit of protease activity was defined as an absorbance of 0.1 at $\text{OD}_{366\text{nm}}$.

Infection of Macrophages with Bacteria

U937-derived macrophages were pretreated with 0.5 mg/ml Hb or PBS for 45 min at 37°C in 5% CO_2 to enable the endocytosis of Hb. The cells were washed once with PBS and then infected at an 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 140 g for 5 min and washing twice with PBS. The cells were then resuspended in PBS containing 2% FBS and maintained for up to 4 h at 37°C . The supernatants were collected and stored at -80°C for cytokine measurements.

Assay for the Colony-Forming Ability of Intracellular Bacteria

To distinguish between extracellular and intracellular bacteria, the cells were infected with *S. aureus* and *S. typhimurium* for 30 min, after which the extracellular bacteria were removed by spinning down cells at 140 g for 5 min (bacteria were not pelleted at this speed) and further washing (twice) with PBS. To exclude potential contamination by extracellular and adherent bacteria, we treated the cells with 50 $\mu\text{g}/\text{ml}$ gentamycin prior to assessment of the intracellular bacterial load. The number of viable intracellular bacteria was quantified by lysis of the infected U937 macrophages with 0.2% Triton X-100 for 10 min. 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, 3 drops of 10 μl each were applied onto a quadrant of the LB agar plate. The plates were incubated at 37°C overnight.

Staining of intracellular *S. aureus* or *S. typhimurium* in Macrophages

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 methods similar to those mentioned previously 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 an LSM 510 META confocal microscope (Zeiss) at $\times 100$ (oil immersion objective). z-stacking at 0.48 μm interval was done to ensure the intracellular localization of the bacteria on the same plane as the cells.

Flow Cytometric Analysis of Hb and Bacterial Uptake by Macrophages

In order to analyze the uptake of Hb and bacteria in individual cells, 1×10^6 U937-derived macrophages pretreated with 0.5 mg/ml FITC-Hb for 45 min were infected with BacLight Red-stained *S. aureus* or *S. typhimurium* (described above) at an MOI of 10 for 30 min. The remnant extracellular bacteria were killed using 50 $\mu\text{g}/\text{ml}$ gentamycin and the cells were analyzed for both Hb and bacterial uptake using the FITC and PE channels, respectively, on a CyAn ADP flow cytometer (Dako). The cells were gated using single stained controls and at least 10^4 cells were analyzed individually for each treatment. The numbers represent the percentages of cells in each quadrant.

Assays for Hb Endocytosis by CD163+ U937-Derived Macrophages

Immunofluorescence Assay

U937-derived macrophages (10^6) were seeded onto coverslips in 24-well plates. Hb (0.5 mg/ml) was incubated with the cells for up to 60 min. The excess Hb was removed by washing twice in PBS. The cells were fixed in 4% paraformaldehyde 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 and CD163 were costained using rabbit anti-Hb primary antibody (1:200 dilution) and goat anti-CD163 (1:200 dilution). Alexa488-conjugated chicken anti-rabbit secondary antibody (1:250 dilution) and NL557-conjugated donkey anti-goat secondary antibody (1:250 dilution) were respectively used to detect the bound primary antibodies. The samples were mounted onto slides using ProLong Gold antifade mounting medium (Invitrogen) containing DAPI and viewed using an LSM510 META confocal microscope under a $\times 100$ oil immersion objective.

Flow Cytometry

U937-derived macrophages (10^6) were incubated with 0.5 mg/ml Hb for 45 min and the excess Hb was removed by washing twice with PBS. The cells were fixed in 4% paraformaldehyde 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 Alexa488-conjugated secondary antibody and analyzed on the FITC channel (λ_{ex} : 490 nm, λ_{em} : 525 nm) on a CyAn ADP flow cytometer (Dako).

Measurement of Mitochondrial ROS Production Using MitoSOX Red

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).

Chemiluminescent-Based Detection of Hb-POX Activity and O_2^- Production

The generation of ROS (O_2^-) by Hb was monitored by the chemiluminescence of the cypridina luciferin analog [29] using a GloMax 20/20 luminometer (Promega). The relative luminescence units per second specifically measure the dynamics of the generation of O_2^- . The slope of the curve was used to calculate the Hb-POX activity.

TMRE Staining to Measure the Mitochondrial Membrane Potential

The mitochondrial membrane potential in live cells was measured using the cell-permeant dye TMRE (Sigma). Briefly, cells were washed once with PBS and incubated with 100 nM TMRE in PBS for 20 min at 37°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 as detected by its fluorescence signal. Cells treated with 10 μ M carbonylcyanide m-chlorophenylhydrazone (CCCP, a mitochondrial uncoupling agent) and 100 μ M hydrogen peroxide (H_2O_2) were used as positive controls to reduce the mitochondrial potential.

MitoTracker Orange Staining of Mitochondria in Macrophages

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

Measurement of Heme Oxygenase-1, p-ERK, and Bax Activation by Flow Cytometry

U937-derived macrophages (3×10^6) 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/PBS 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 anti-heme oxygenase-1 (1:200), anti-pERK (1:100), or anti-Bax (1:50) (Cell Signaling) and Alexa488-conjugated secondary antibody (1:400) (chicken anti-rabbit; Invitrogen) followed by 2 washes after incubation with the primary antibody. The cells were then washed 4 times with PBS, and 10,000 cells in the gated region were acquired and analyzed on a CyAn ADP flow cytometer (Dako). Cells stained with the secondary antibody alone served as the negative control.

Preparation of the Cell Lysate and Immunoblotting

Cultured cells were harvested and pelleted, and protein extraction was performed in ice-cold RIPA lysis buffer (Pierce) containing 1 mM PMSF and 1 $\times$ protease inhibitor cocktail (Sigma). Thirty micrograms of total protein were resolved by 12% SDS-PAGE and then electrotransferred to a polyvinylidene difluoride membrane in Tris-glycine buffer with 20% methanol. Membranes were probed with a rabbit monoclonal antibody against p-ERK or p-p38 MAPK (Cell Signaling) overnight followed by goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Dako). For the loading control, blots were probed with a mouse monoclonal antibody against GAPDH (Santa Cruz), followed by goat anti-mouse HRP-conjugated secondary antibody (Dako). The blots were stripped and reprobed with rabbit anti-Bax (Cell Signaling) and mouse anti-Bcl2 (Santa Cruz). Bands were visualized with a SuperSignal chemiluminescence substrate (Pierce).

Tricine SDS-PAGE and Western Blotting to Visualize Hb Proteolysis

The cleaved fragments released upon the proteolytic cleavage of Hb by V8 protease from *S. aureus* were resolved using 10% Tricine-SDS-PAGE under reducing conditions according to the methods described by Schagger [30]. The protein bands were stained with Coomassie Blue. Western blotting was used to analyze the Hb degradation in Hb-treated macrophages upon infection with *S. aureus* or *S. typhimurium*. Briefly, 50 μ g total protein from the cell lysate were resolved on 10% Tricine gel and electrotransferred to a polyvinylidene difluoride membrane in Tris-glycine buffer with 20% methanol. The membrane was probed with a rabbit-polyclonal antibody against Hb (Sigma) overnight followed by an HRP-conjugated goat anti-rabbit secondary antibody. For the loading control, blots were probed with a rabbit antibody against actin (Sigma) followed by an HRP-conjugated goat anti-rabbit secondary antibody. The protein bands were visualized with SuperSignal chemiluminescence substrate (Pierce).

Quantification of Cytokines by ELISA

The levels of TNF- α and IL-10 in the culture supernatants were measured using commercially available kits (OptEIA Human TNF- α and IL-10 ELISA Kits; BD Biosciences) according to the manufacturer's instructions.

Annexin V and 7-AAD Staining of Apoptotic Cells

Staining of apoptotic cells was performed using an Annexin V-FITC Apoptosis Detection Kit (eBioscience) and 7-AAD Viability Staining Solutions (eBioscience) according to the manufacturer's instructions. Briefly, cells were washed successively with PBS and 1 $\times$ 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. 7-AAD was added at a dilution of 1:20 to the cell suspension and immediately analyzed on a CyAn ADP flow cytometer (Dako). Staurosporine treated cells (1 μ M) were used as the positive control. Annexin V-positive-stained cells were scored as apoptotic. Single stained controls were used to gate the different cell populations.

Cell Viability Assay

U937-derived macrophages (0.5×10^5) were seeded in 96-well plates and infected with *S. aureus*, *S. typhimurium*, or *P. aeruginosa* in the presence or absence of preconditioned supernatants. The viability was then determined by incubating the cells with CellTiter-Blue (Promega) dye for 2 h. The nonfluorescent dye is metabolized within live cells to form a fluorescent product, which was measured at λ_{ex} 560 nm/ λ_{em} 590 nm using a microplate reader (BioTek).

Caspase Activity Assay

The activities of caspase-3 and caspase-9 were measured by resuspending the cell pellets in 30 μ l phipilux-G $_2$ D $_2$ and caspalux 9-M $_2$ D $_2$ substrate (OncoImmunin) and incubating them for 40 min at 37°C. After washing in FACS buffer, the cells were analyzed at λ_{ex} 552 nm/ λ_{em} 580 nm.

Statistical Analysis

Data represent the means $\pm$ SEM of at least 2 independent experiments, each conducted in triplicate. $p < 0.05$ was considered statistically significant via an ANOVA test with a 95% confidence limit ($\alpha = 0.05$). Post hoc analysis was performed using Turkey's honestly significant difference (HSD) test at $\alpha = 0.05$.

Results

Hb Priming Enhances the Intracellular Survival of Bacteria in Macrophages

To study the effect of Hb uptake by macrophages on the intracellular survival of bacteria, we preincubated U937-derived macrophages with 0.5 mg/ml Hb over a time course of up to 60 min. Extracellular Hb concentrations are known to reach up to 0.6 mg/ml in sickle cell anemia and 0.5 mg/ml during sepsis [7]. Here, we used 0.5 mg/ml Hb to mimic severe hemolysis induced during sepsis. We observed the maximal uptake of Hb by the CD163+ macrophages at 45 min and hence we used this time period in further experiments (online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000258236). The cells were subsequently infected for 30 min with either *S. aureus* (Gram positive) or *S. typhimurium* (Gram negative). Since 30 min is usually employed for the phagocytosis of *S. aureus* [12] and *S. typhimurium* [31] into human macrophages, we used this time period consistently in our experiments. The remaining extracellular bacteria were removed by centrifugation and washing (see Materials and Methods) 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 via colony-forming unit (CFU) assay of the cell lysate. At 4 h postinfection, the Hb-primed macrophages harbored nearly twice ($p < 0.05$) the number of intracellular bacteria compared to unprimed control macrophages which were also corticosteroid differentiated (see Materials and Methods) but treated with only serum-supplemented PBS (fig. 1a). 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 levels of viability inside the U937 cells. To exclude potential contamination by extracellular and adherent bacteria, we treated the cells with 50 µg/ml gentamycin [32] prior to assessment of the intracellular bacterial load. Consistent with our earlier results (fig. 1a), we observed that Hb-primed macrophages possessed higher numbers of intracellular *S. aureus* and *S. typhimurium* in a time-dependent manner compared to unprimed control macrophages (online suppl. fig. S2a). Importantly, the effect of Hb priming on the intracellular bacterial load was dependent on the dose of Hb (online suppl. fig. S2b). Since 0.5 mg/ml Hb induced the highest growth/survival of intracellular bacteria, we used

this concentration of Hb consistently. To corroborate our observation that the increased intracellular bacterial load in Hb-primed macrophages was due to the endocytosed Hb, U937-derived macrophages were left untreated or pretreated with 0.5 mg/ml FITC-conjugated Hb for 45 min and then infected with BacLight Red-stained *S. aureus* or *S. typhimurium*. After removal of the extracellular bacteria using 50 µg/ml gentamycin, the extent of Hb and bacterial uptake was quantitated in individual cells using flow cytometry. Results showed a distinct population of cells which were double positive for Hb as well as internalized *S. aureus*/*S. typhimurium* (red boxes) upon infection of Hb-primed macrophages, which was absent in control untreated infected cells (online suppl. fig. S2c), affirming that indeed cells which endocytosed Hb also had a higher bacterial load.

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. In agreement with the results shown in figure 1a, we consistently observed a time-dependent increase in the mean number of *S. aureus* and *S. typhimurium* per cell after Hb priming (fig. 1b, c). z-stack images were acquired to ensure that only the intracellular bacteria were quantified (online suppl. fig. S3). Taken together, our results suggest that the Hb endocytosed by the macrophages supports the intracellular growth and persistence of bacteria during infection.

Infection of Hb-Primed Macrophages Resulted in Downregulation of Hb-POX Activity and Mitochondrial Stress

Redox-active Hb has been reported to aggregate and induce cytotoxicity in macrophages [33]. Since we observed a higher intracellular bacterial count in Hb-primed macrophages (fig. 1), it was pertinent to understand how they modulate Hb-driven toxicity in macrophages in order to establish survival. Hence, we measured Hb-POX activity with and without the addition of *S. aureus* or *S. typhimurium* in vitro. The results showed that Hb-POX activity was significantly reduced upon incubation with *S. aureus* or *S. typhimurium* (fig. 2a). Incubation with the ROS scavenger N-acetyl cysteine (NAC) was used as a positive control. Negative controls such as *S. aureus* or *S. typhimurium* alone (without Hb) produced only baseline readings. Next, we measured the mitochondrial ROS production in Hb-treated macrophages with or without infection by *S. aureus* and *S. typhimurium* at 4 h postinfection, a time during which we had earlier observed the

maximal intracellular bacteria (fig. 1). Stimulation with Hb alone increased the mitochondrial ROS production in a dose-dependent manner in macrophages compared to control cells treated with only serum-supplemented PBS (fig. 2b). 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 ($p < 0.05$) compared to control unprimed infected cells, suggesting that the intracellular-resident bacteria actively downregulated or effectively quenched the released ROS.

At this juncture, we examined the activation of Hb-POX activity by the microbial proteases in 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 [34]. However, we only observed a weak activation (≤ 2 fold) of Hb-POX activity by *S. aureus* PC1839 (online suppl. fig. S4a), unlike proteases like subtilisin A from *Bacillus subtilis*, which has been reported to induce up to a 4-fold increase in Hb-POX activity [11]. Furthermore, *S. typhimurium*, which is also hemolytic [35], showed only a weak activation of Hb-POX activity. In addition, there was no significant dose-dependent effect of the proteases from *S. aureus* or *S. typhimurium* on Hb-POX activity.

Next, we tested whether the mode of action of the intracellular bacteria was to degrade Hb and utilize it for their own survival. Hence, we analyzed Hb degradation by the *S. aureus* V8 protease using 10% Tris-Tricine SDS-PAGE. Upon incubation with the V8 protease from *S. aureus* (strain PC1839) [36], Hb was cleaved and degraded in a dose-dependent manner (online suppl. fig. S4b, red boxes). This is supported by the release of ~ 10 kDa Hb peptides which are only produced by the V8+ *S. aureus* PC1839 but not by the control V8- *S. aureus* pALC1420. Consistent with this result, we also observed Hb degradation in macrophages upon infection with *S. aureus* or *S. typhimurium* (online suppl. fig. S4c).

To analyze whether the intracellular *S. aureus* and *S. typhimurium* in Hb-primed macrophages degraded Hb

to free heme, free iron, and globin chains/peptides, we measured the activation of the Hb-catabolizing enzyme heme oxygenase-1 (HO-1) [37]. The results showed that infection of Hb-primed macrophages with *S. aureus* or *S. typhimurium* induced higher HO-1 levels compared to control unprimed infected cells (online suppl. fig. S4d). Hence, we propose that both *S. aureus* and *S. typhimurium* may degrade the endocytosed Hb and utilize the liberated heme iron to support their intracellular survival without eliciting a strong Hb-POX cycle, which would have generated microbicidal ROS.

Next, we examined the effect of Hb-induced ROS production on mitochondrial pore permeability at 4 h postinfection on the basis of the interrelationship between ROS and mitochondrial depolarization [38]. Figure 2c shows that, compared to the serum-supplemented PBS control, Hb stimulation caused a time-dependent decrease in 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 Hb-POX activation and ROS production (fig. 2a, b). This consistently indicates that intracellular bacteria suppress Hb-POX activity and hence lessen mitochondrial depolarization. Taken together, these results suggest that intracellular-resident bacteria downregulate Hb-induced mitochondrial stress in order to survive within the cells.

Hb-Primed Macrophages Infected by S. aureus or S. typhimurium Show Downregulated MAPK-Bax Signaling

In order to elucidate the mechanism underlying how infection by *S. aureus* and *S. typhimurium* might modulate the host signaling pathways in Hb-primed macrophages, we measured the key signaling molecules at 4 h postinfection. The results showed that while Hb stimulation upregulated the expression of Bax, Hb-primed cells infected with *S. aureus* and *S. typhimurium* abrogated Bax (fig. 3a, red boxes; online suppl. fig. S5). In contrast,

Fig. 1. Hb priming enhances the intracellular survival of bacteria in macrophages. **a** U937-derived macrophages (0.5×10^6) were either left untreated or pretreated with 0.5 mg/ml Hb for 45 min and then infected with *S. aureus* or *S. typhimurium* for 30 min (MOI 10). The lysates at the indicated time points were serially diluted onto agar plates overnight and the number of CFU was calculated. * $p < 0.05$. **b** Confocal microscopy to track the intracellular replication of *S. aureus* or *S. typhimurium* (red) in primary monocyte-derived macrophages at the indicated time points with

or without pretreatment with 0.5 mg/ml Hb for 45 min. Images were acquired using an LSM510 META confocal microscope under a $\times 100$ oil immersion objective. Scale bars = 5 μ m. **c** The mean number of intracellular bacteria per infected macrophage at the indicated time points was quantified by microscopy and verified to be within cell boundaries using z-stack images. * $p < 0.05$. The significance of the difference in mean intracellular bacterial loads in unprimed and Hb-primed cells was verified using Turkey's HSD test. *S.a* = *S. aureus*; *S.t* = *S. typhimurium*.

(For figure 1 see next page.)

a

c

b

Fig. 2. Infection of Hb-primed macrophages resulted in the down-regulation of Hb-POX activation and mitochondrial stress. **a** Hb-POX activity was measured upon incubation with *S. aureus* or *S. typhimurium* for 30 min using the CLA-CL assay. * $p < 0.05$, ** $p < 0.005$ using Turkey's HSD test. **b** Mitochondrial ROS in U937-derived macrophages. U937-derived macrophages (0.5×10^6) were loaded with $5 \mu\text{M}$ MitoSOX Red and either left untreated or pretreated with 0.5 mg/ml Hb for 45 min. The cells were then infected with *S. aureus* or *S. typhimurium* (MOI 10) and the fluorescence intensity of the dye was quantitated using a CyAn ADP

flow cytometer. * $p < 0.05$ using Turkey's HSD test. **c** Mitochondrial depolarization in U937-derived macrophages. TMRE staining to measure the mitochondrial pore permeability in U937 macrophages infected with either *S. aureus* or *S. typhimurium* (MOI 10) with or without pretreatment with 0.5 mg/ml Hb for 45 min. CCCP ($10 \mu\text{M}$)- and H_2O_2 ($100 \mu\text{M}$)-treated cells were used as positive controls. * $p < 0.05$ using Turkey's HSD test. **a–c** Untreated refers to differentiated U937 macrophages treated with PBS supplemented with 2% serum. S.a = *S. aureus*; S.t = *S. typhimurium*.

Fig. 3. Hb-primed macrophages infected with *S. aureus* or *S. typhimurium* show downregulated MAPK-Bax signaling. **a** Western blot analysis of p-p38, p-ERK, Bax, and Bcl2 in Hb-only-treated and Hb-pretreated U937 macrophages infected with *S. aureus* or *S. typhimurium*. The red boxes show the downregulation of p-ERK, p-p38, and Bax and the maintenance of Bcl2 in infected Hb-pretreated macrophages. GAPDH was used as a loading control. Full-length blots are presented in online supplementary fig-

ure S5. **b** FACS 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*. **c** Cytokine production (TNF- α and IL-10) in Hb-only-treated and Hb-pretreated U937 macrophages infected with *S. aureus* or *S. typhimurium*. * $p < 0.05$, ** $p < 0.005$ using Turkey's HSD test. S.a = *S. aureus*; S.t = *S. typhimurium*; Bac = bacteria.

(For figure 3 see next page.)

cells infected without prior Hb priming showed a sustained level of Bax, implying that Hb priming, which produced a 2-fold increase in the intracellular bacterial count (fig. 1a), 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 (fig. 3a), suggesting that the intracellular-resident bacteria kept the host cells alive in order to survive within these Hb-loaded cells.

Since p-ERK and p-p38 MAPK signaling are essential for the downstream activation of Bax [39], we queried whether the intracellular-resident bacteria in Hb-primed macrophages would manipulate the MAPK signaling pathway. Figure 3a and b shows that, while stimulation with Hb alone upregulated both p-p38 and p-ERK signaling, Hb-primed macrophages infected with *S. aureus* and *S. typhimurium* downregulated both p-p38 and p-ERK. This result is consistent with the downregulation of Bax and the maintenance of Bcl2 levels upon infection of 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- α (pro-inflammatory) and IL-10 (anti-inflammatory) at 6 h postinfection of Hb-primed macrophages. Figure 3c shows that stimulation with Hb alone significantly ($p < 0.005$) upregulated the proinflammatory cytokine TNF- α compared to the anti-inflammatory IL-10. On the contrary, infection of Hb-primed macrophages by *S. aureus* or *S. typhimurium* induced a higher IL-10 production, suggesting that the intracellular-resident bacteria skewed the cytokine production towards an anti-inflammatory phenotype.

In order to understand how infection by extracellular pathogens impacts host cell survival with or without Hb priming, we used *P. aeruginosa*, a Gram-negative extracellular bacterium [25], as a control to study its effect on MAPK-Bax signaling and cytokine production. Hb-primed macrophages infected with *P. aeruginosa* showed upregulation of Bax and p-ERK signaling compared to control unprimed infected cells with a concomitantly higher production of the inflammatory TNF- α (online suppl. fig. S6a, 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.

Hb-Primed Macrophages Harboring S. aureus or S. typhimurium Show Suppressed Apoptosis

Next, we measured the extent of apoptosis of cells at 4 h postinfection with either intracellular bacteria (*S. aureus*/*S. typhimurium*) or extracellular bacteria (*P. aeruginosa*), with or without Hb priming. Figure 4a shows that while 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 which were not primed with Hb. This is consistent with our observation of a higher activation of the MAPK-Bax signaling pathway and, consequently, higher TNF- α production (online suppl. fig. S6a, b). On the contrary, Hb-primed macrophages infected with either *S. aureus* or *S. typhimurium* showed a significant ($p < 0.05$) drop in the level of apoptosis when compared to infected unprimed macrophages. This result is concordant with our earlier finding of a drop in the activation of the MAPK-Bax pathway and a higher IL-10 production as opposed to TNF- α (fig. 3a–c).

To corroborate that the intracellular bacteria in Hb-primed macrophages suppressed mitochondrial stress (fig. 2) and downregulated MAPK-Bax signaling (fig. 3a, b), we investigated caspase activation at 4 h postinfection of Hb-primed macrophages. Figure 4b and c shows that Hb alone induces activation of both the initiator caspase-9 and the executioner caspase-3. However, Hb-primed macrophages infected with either *S. aureus* or *S. typhimurium* downregulated both caspase-9 and caspase-3. In stark contrast, *P. aeruginosa*-infected Hb-primed macrophages showed higher caspase-9 and sustained caspase-3 activation compared to infected unprimed macrophages (online suppl. fig. S6c).

Hb-Primed Cells Challenged with S. aureus or S. typhimurium Promote the Infectivity of Neighboring Cells

To determine whether the anti-inflammatory cytokine production by intracellular bacteria-infected Hb-primed macrophages has an impact on the infectivity and/or 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 recipient cells. Before the transfer, the culture supernatants were centrifuged at 10,000 g for 10 min to remove any cell debris and treated with 50 μ g/ml gentamycin to kill remnant extracellular bacteria. To measure the infectivity on the recipient cells, we challenged the Hb-primed recipient cells with *S. aureus* or *S. typhimuri-*

Fig. 4. Hb-primed macrophages harboring *S. aureus* or *S. typhimurium* show suppressed apoptosis. **a** Apoptosis of Hb-only-treated and Hb-pretreated U937 macrophages infected with *P. aeruginosa*, *S. aureus*, or *S. typhimurium* was quantified by staining with annexin V-FITC and 7-AAD. * $p < 0.05$ using Turkey's HSD test. Caspase-9 (**b**) and caspase-3 activity (**c**) in Hb-only-treated

and Hb-pretreated U937 macrophages infected with *S. aureus* or *S. typhimurium* was measured via caspase-9 and caspase-3 assay, respectively. **a–c** Untreated refers to differentiated U937 macrophages treated with PBS supplemented with 2% serum. *P.a* = *P. aeruginosa*; *S.a* = *S. aureus*; *S.t* = *S. typhimurium*.

Fig. 5. Hb-primed cells challenged with *S. aureus* or *S. typhimurium* promote the infectivity of neighboring cells. **a** CFU assay showing the intracellular bacterial load (*S. aureus*/*S. typhimurium*) in U937-derived macrophages upon supplementation with supernatants from macrophages stimulated with Hb alone, *S. aureus*/*S. typhimurium*/*P. aeruginosa* alone, or Hb-primed cells infected with *S. aureus*/*S. typhimurium*/*P. aeruginosa*. Cell viability (**b**) and cytokine production (**c**) by recipient cells upon supernatant transfer from Hb-alone- and *S. aureus*/*S. typhimurium*/*P. aeruginosa*-infected cells with or without Hb priming. **b** The y-axis indicates the fluorescence (560/590 nm) of the CellTiter-Blue dye. * $p < 0.05$, ** $p < 0.005$ using Turkey's HSD test. *P.a* = *P. aeruginosa*; *S.a* = *S. aureus*; *S.t* = *S. typhimurium*; Unt = untreated cells; Sup = supernatant. Untreated refers to differentiated U937 macrophages treated with PBS supplemented with 2% serum.

um. Cells treated with the supernatants from Hb-primed cells that had been challenged with *S. aureus* or *S. typhimurium* possessed higher intracellular bacterial loads (fig. 5a) compared to the 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*). This is consistent with our previous results (fig. 3c) showing a higher anti-inflammatory IL-10 production by *S. aureus*- and *S. typhimurium*-infected Hb-primed macrophages. Compared to all of the control supernatants, we also recorded a significantly higher host cell viability and a higher anti-inflammatory IL-10 pro-

duction ($p < 0.005$) in recipient cells upon the transfer of supernatants from Hb-primed cells that had been challenged with *S. aureus*/*S. typhimurium* (fig. 5b, c). This is in agreement with earlier findings.

In conclusion, we have demonstrated a molecular mechanism that explains the preferential intracellular survival of bacteria in Hb-primed macrophages without eliciting strong Hb-POX activity. Such intracellular bacteria thrive by downregulating the ERK- and p-p38-mediated activation of Bax and downstream caspases to silently persist in host cells while suppressing apoptosis (fig. 6).

Fig. 6. 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 the 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 internalized into the macrophages, remain in functional contact with each other. The intracellular bacteria downregulate the Hb-redox activity and at the same time induce the Hb-catabolizing enzyme HO-1 to feed on the liberated heme iron for growth and survival. Subsequently, the bacteria downregulate p-p38/p-ERK-mediated activation of the proapoptotic 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.

Discussion

Hemolysis releases prooxidative and cytotoxic Hb [5] into the plasma. Phagocytic cells scavenge and uptake plasma Hb via receptor-mediated endocytosis, preempting infection by microbial invaders which sequester heme iron. However, during an infection of these Hb-loaded cells by intracellular bacteria, both the endocytosed Hb and the pathogen could be in functional contact with each other. Consistently, hemolytic patients are known to exhibit increased susceptibility to intracellular bacterial infections, although the underlying molecular mechanism remains unclear. Here, our findings demonstrate that hemolytic bacteria such as *S. aureus* and *S. 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 [12].

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 has been known that pathogen-mediated proteolysis of host proteins provides a source of amino acids critical for the survival of the microbe [40]. However, we found that intracellular bacteria limit the

activation of Hb-POX to allow its replication while evading the microbicidal ROS production (fig. 2a).

Apart from its classical role in cell proliferation and differentiation, MAPK signaling (e.g. ERK and p-p38) has also been implicated in the induction of apoptosis both in vitro and in vivo [41, 42]. In particular, ERK activation has been associated with cell death induced by ROS [43]. We found that Hb-generated ROS induced the activation of p-p38 and ERK and, consequently, the apoptosis of macrophages. However, upon infection of Hb-primed macrophages with opportunistic intracellular bacteria (represented by *S. aureus* and *S. typhimurium* in this study), we observed a reduction in ERK-induced activation of the proapoptotic Bax and downstream caspases while the level of the antiapoptotic Bcl2 was maintained (fig. 3, 4). On the other hand, compared to control unprimed infected cells, Hb-primed macrophages infected by extracellular bacteria (*P. aeruginosa*) predominantly upregulated ERK-mediated activation of the proapoptotic Bax and caspase (online suppl. fig. S6a, c). Hence, it is possible that Hb priming dictates differential effects on host cell survival depending on the nature of the infection.

Consequent to inhibition of proapoptotic signaling, intracellular bacteria also skewed the cytokine profile in Hb-primed macrophages towards an anti-inflammatory (IL-10) phenotype rather than the proinflammatory TNF- α (fig. 3), probably to counter apoptosis (fig. 4) and

promote infectivity in neighboring cells (fig. 5). Nevertheless, the suppression of apoptosis by the intracellular bacteria was only partial, likely due to the parallel activation of Toll and Nod-like receptors [44], implicating the potential homeostatic shift towards death when the phagocyte becomes overburdened with bacteria.

In summary, our findings provide mechanistic insight into how hemolytic bacteria may exploit the Hb-scavenging mechanism to preferentially survive intracellularly within the Hb-primed phagocytes, subtly utilizing the heme iron without eliciting strong POX activity (escaping from microbicidal ROS) and suppressing the apoptosis of the host cell.

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Disclosure Statement

The authors declare no competing financial interests.

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