Malaria impairs resistance to *Salmonella* through heme- and heme oxygenase–dependent dysfunctional granulocyte mobilization

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In sub-Saharan Africa, invasive nontyphoid *Salmonella* (NTS) infection is a common and often fatal complication of *Plasmodium falciparum* infection. Induction of heme oxygenase-1 (HO-1) mediates tolerance to the cytotoxic effects of heme during malarial hemolysis but may impair resistance to NTS by limiting production of bactericidal reactive oxygen species. We show that co-infection of mice with *Plasmodium yoelii* 17XNL (Py17XNL) and *Salmonella enterica* serovar Typhimurium 12023 (*Salmonella typhimurium*) causes acute, fatal bacteremia with high bacterial load, features reproduced by phenylhydrazine-induced hemolysis or hemin administration. *S. typhimurium* localized predominantly in granulocytes. Py17XNL, phenylhydrazine and hemin caused premature mobilization of granulocytes from bone marrow with a quantitative defect in the oxidative burst. Inhibition of HO by tin protoporphyrin abrogated the impairment of resistance to *S. typhimurium* by hemolysis. Thus, a mechanism of tolerance to one infection, malaria, impairs resistance to another, NTS. Furthermore, HO inhibitors may be useful adjunctive therapy for NTS infection in the context of hemolysis.

NTS bacteremia is the most common cause of community-acquired bacteremia in many parts of sub-Saharan Africa, and NTS co-infection has been associated with high malaria mortality. The association of NTS infection with hemolysis is well established in humans with malaria (especially severe malarial anemia) and sickle cell disease and in mice with hemolysis resulting from rodent malaria infection, treatment with phenylhydrazine or erythrocyte-targeting antibodies, or red blood cell enzyme defects. It has been assumed that hemolysis-induced macrophage dysfunction is responsible for this phenomenon, although there is no direct evidence that macrophages are the primary refuge of NTS in vivo in the context of hemolysis.

Hemolysis results in liberation of heme, leading to expression of the inducible isoform of HO (HO-1), which degrades heme to biliverdin, carbon monoxide and iron. Heme is pro-oxidant, induces neutrophil migration and activates the neutrophil oxidative burst, but HO-1 (and its products) are essential in cytoprotection (reviewed in ref. 17), as evidenced by the severe susceptibility to oxidative stress of mice and humans with HO-1 deficiency. HO-1 induction has been shown to protect against infectious, inflammatory and hypoxic-ischemic insults in mice (reviewed in ref. 21) and has been linked to modulation of malarial pathogenesis and sickle cell disease.

Recently, in mice, induction of HO-1 has been proposed as a tolerance mechanism in severe malaria and polymicrobial sepsis; HO-1 lessens heme-mediated tissue damage and enhances survival without reducing pathogen load. A key cytoprotective effect of HO-1, and thus a likely explanation for its ability to confer tolerance, is its ability to limit the production of damaging reactive oxygen species (ROS, reviewed in ref. 17). However, ROS are crucial for resistance to certain pathogens, including *Salmonella* species, and this raises the possibility that tolerance of one pathogen may sometimes come at the price of loss of resistance to another. We hypothesized that liberation of heme by intravascular hemolysis may lead to HO-1 induction and impairment of resistance to NTS, with increased bacterial replication and mortality.

**RESULTS**

Hemolysis and heme impair resistance to *S. typhimurium* bacteremia

To determine whether heme liberated by hemolysis impairs resistance to NTS infection, we compared survival and bacterial loads after intraperitoneal infection of C57BL/6 mice with GFP-expressing *S. typhimurium* with or without preceding Py17XNL co-infection, phenylhydrazine (PHZ) or hemin treatment. Py17XNL infection of C57BL/6 mice caused a self-resolving infection; parasitemia peaked at 20–30% and was accompanied by progressive hemolytic anemia (Fig. 1a). By contrast, PHZ treatment caused acute hemolysis (Fig. 1b). In both cases, plasma heme concentrations were markedly increased and similar to concentrations achieved 12 h after injection of hemin (Fig. 1c), but without depletion of haptoglobin or hemopexin.

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**Figure 1** Hemolysis and heme are associated with impaired resistance to *S. typhimurium*. (a) Erythrocyte count and parasitemia of mice infected with Py17XNL. Data are representative of seven independent experiments (mean ± s.d. of 5–25 mice per time point). (b) Erythrocyte count before and 18 h after subcutaneous injection of PHZ. Data are representative of three independent experiments (mean ± s.d. of five mice). (c) Plasma heme concentration during Py17XNL infection and 15 h after PHZ treatment or 12 h after hemin treatment. Data are representative of at least two independent experiments (mean ± s.d. of four or five mice) per condition and time point. (d) Survival (time until reaching humane endpoint) of mice infected with *S. typhimurium* on day 15 after Py17XNL infection or 6 h after PHZ, first dose of hemin or PBS treatment. Data are representative of at least four independent experiments (mean ± s.d. of four or five mice) per condition. (e) *S. typhimurium* bacterial loads in whole blood, spleen, liver and bone marrow 18 h after infection for PBS-treated controls and at humane endpoint for other conditions. (f) *S. typhimurium* bacterial loads in whole blood, spleen and liver at 18 h or 72 h (humane endpoint) after infection for PBS-treated mice. In (e,f), data are representative of at least two independent experiments (mean ± s.d. of four or five mice) per condition. Significance determined by two-tailed paired Student’s *t* test (b), one-way ANOVA with Dunnett’s multiple comparison test (c,e,f), or log-rank Mantel Cox test (d). *P* < 0.05, **P** < 0.01, ***P** < 0.001. **In contrast, neutrophils from PBS-treated mice showed mature nuclear morphology and did not contain *S. typhimurium*.**

The accumulation of GFP* bacteria in granulocytes was not simply due to failure of bacterial uptake by monocytes and macrophages, as the proportion of GFP* cells in the spleen that were either monocytes or macrophages (F4/80hiCD11bhi and F4/80hiCD11blow, respectively) did not differ between Py17XNL-infected or PHZ- or hemin-treated mice and those treated with PBS alone (Supplementary Fig. 2c).

**Py17XNL inhibits granulocyte oxidative burst and bacterial killing**

As there is no obvious defect in uptake of *S. typhimurium* by macrophages and monocytes after hemolysis or hemin treatment, accumulation of *S. typhimurium* in blood granulocytes may result from impaired bacterial killing or a more permissive intracellular environment for bacterial replication. To investigate these possibilities, we isolated CD11b+ cells from blood of Py17XNL-infected and uninfected mice and compared their ability to phagocytose and kill *S. typhimurium*. Neither flow cytometric analysis of GFP* cells nor quantitative culture (in a gentamicin protection assay) revealed any differences in the rates of phagocytosis of *S. typhimurium* between neutrophils or monocytes or between cells from malaria-infected or uninfected mice (Fig. 3a,b); we confirmed the intracellular location of GFP* bacteria by confocal microscopy (Supplementary Fig. 3a,b). However, when we lysed cells after 2 h in the gentamicin protection assay and enumerated live bacteria by culture, the live bacterial recovery from cells from Py17XNL-infected mice was significantly higher than from control mice (Fig. 3b), indicating considerable impairment of intracellular killing of *S. typhimurium* by cells from Py17XNL-infected mice.

As HO-1 reduces the production of ROS and 32,33, and as phagocyte NADPH oxidase is essential for resistance to *S. typhimurium* early in infection34, we investigated whether Py17XNL infection impairs the granulocyte oxidative burst. Using oxidation of dihydroxydamine to its fluorescent derivative rhodamine as a readout for oxidative burst34, we observed progressive suppression of the phorbol myristate acetate (PMA)-induced oxidative burst of blood granulocytes during

(Supplementary Fig. 1a,b). Survival of Salmonella-infected mice was dramatically shortened by prior Py17XNL infection, PHZ or hemin treatment (Fig. 1d) and was significantly shorter in PHZ- and hemin-treated mice (16 h) than in Py17XNL co-infected mice (18 h) (P < 0.01, log-rank Mantel Cox test). PHZ, hemin and Py17XNL did not cause any mortality in the absence of *S. typhimurium* infection (data not shown).

Decreased survival of malaria-infected, PHZ-treated or hemin-treated mice after *S. typhimurium* infection was accompanied by higher bacterial loads in whole blood, spleen, liver and bone marrow (Fig. 1e), and bacteremia was much more pronounced; immediately before death (that is, 16–18 h after infection in Py17XNL-infected, PHZ- or hemin-treated mice and 72 h after infection in control mice), bacterial loads in the blood of infected or treated mice were proportionately higher, and bacterial loads in liver and spleen proportionately lower (Fig. 1e), than in control mice (Fig. 1f).

*S. typhimurium* localize in granulocytes following hemolysis

By flow cytometry, we identified GFP* (*S. typhimurium*-containing) cells in blood, spleen and bone marrow. In the blood of Py17XNL-infected mice, and of PHZ- or hemin-treated mice, just before death, we found salmonellae predominantly in Gr-1hi cells (Fig. 2a), and they were enriched in this cell population compared to the bacteria in control (PBS-treated) mice (Fig. 2b). The proportion of all GFP*Gr-1hi cells in blood, spleen and bone marrow (Fig. 2c) correlated with the bacterial load determined by culture (Fig. 1e,f). We identified the Gr-1hi cells as granulocytes (Ly6GhiF4/80hiCD115; Supplementary Fig. 2a,b). Almost all GFP* cells were CD115hi (Fig. 2d); moreover, *S. typhimurium* infection caused a higher proportion of Gr-1hiCD115hi blood leukocytes (Fig. 2c), suggesting that immature granulocytes are mobilized from the bone marrow to the peripheral blood during infection39. In support of this, blood films from Py17XNL-infected and PHZ- or hemin-treated mice 18 h after *S. typhimurium* infection showed numerous neutrophils containing *S. typhimurium*, and many of these neutrophils had immature nuclear morphology (Fig. 2f).
**infection (Fig. 3c).** Granulocytes were not simply refractory to PMA stimulation, as PMA-induced degranulation (assessed by surface CD11b expression) was actually enhanced 14 and 21 d after malaria infection (Fig. 3d). In contrast, blood granulocytes isolated 24 h after PHZ or hemin treatment did not differ from those of control mice in oxidative burst capacity, degranulation, or killing of S. typhimurium (Fig. 3c,d and Supplementary Fig. 3c,d).

**Hemolysis induces dysfunctional granulocyte mobilization**

Accumulation of heme after PHZ-mediated hemolysis or hemin administration is faster than during Py17XNL infection. Heme directly induces neutrophil migration and ROS production, whereas the subsequent HO-1 induction in myeloid cells can suppress maturation and oxidative burst activity. As HO-1 is induced in bone marrow by hemolysis, we wondered whether the chronic hemolysis associated with Py17XNL infection might induce HO-1 expression in immature bone marrow myeloid cells, suppress their oxidative burst capacity as they mature and allow gradual accumulation of dysfunctional cells in the circulation, as opposed to acute hemolysis (induced by PHZ), which may both activate the oxidative burst of circulating granulocytes and mobilize functionally immature bone marrow granulocytes, resulting in heterogeneous oxidative burst activity of blood granulocytes (as suggested by Fig. 3c).

In mice, granulocyte maturation in bone marrow is characterized by increasing expression of Gr-1 (ref. 29). Gr-1<sup>hi</sup> cells are mature neutrophils, and Gr-1<sup>lo</sup> cells are immature granulocytes and granulocyte progenitors; the Gr-1<sup>int</sup> (intermediate) compartment contains a mixture of cell types. Generation of an oxidative burst is restricted to a functionally mature subpopulation of cells<sup>36</sup> in the Gr-1<sup>hi</sup>, and to a lesser extent the Gr-1<sup>int</sup>, compartments (Supplementary Fig. 4).

Treatment with hemin or PHZ and Py17XNL infection all caused marked depletion of Gr-1<sup>hi</sup> cells from bone marrow (Fig. 3e and Supplementary Fig. 5). For PHZ and hemin treatment, loss of Gr-1<sup>hi</sup> cells from bone marrow was accompanied by an increase in granulocytes in peripheral blood (Supplementary Fig. 5), confirming the effect of free heme in mobilization of granulocytes from bone marrow to the periphery. Although the proportion of circulating granulocytes did not increase during Py17XNL infection, granulocyte mobilization might have been obscured by an overall increase in leukocyte count or granulocyte redistribution (for example, from blood to the spleen). S. typhimurium infection caused granulocyte mobilization in PBS-treated mice and markedly exacerbated the granulocyte mobilization in Py17XNL-infected and PHZ- or hemin-treated mice (Fig. 3e and Supplementary Fig. 5), consistent with the presence of immature granulocytes in blood (Fig. 2e). To confirm that hemolysis and bacterial challenge did indeed result in granulocytes with reduced oxidative burst activity entering the circulation, we assessed the oxidative burst of circulating granulocytes. Eight hours after S. typhimurium infection, the oxidative burst response to PMA was enhanced in PBS-treated mice (presumably due to priming), but the oxidative burst capacity was markedly lower in PHZ-treated mice (Fig. 3f).
Finally, we investigated whether maturation of the oxidative burst in bone marrow granulocytes was also impaired. On day 14 of Py17XNL infection, and 18 h after PHZ or hemin treatment, there was a clear quantitative defect in the PMA-induced oxidative burst of Gr-1hi cells (Fig. 3g), evident as an increase in the proportion of cells with low oxidative burst capacity and a decrease in the proportion of cells with high burst capacity (Fig. 3h), compared to the PBS control.

Together, these data indicate that intravascular heme (released during hemolysis) mobilizes granulocytes from bone marrow and simultaneously impairs development of their oxidative burst. Thus, granulocytes entering the circulation in response to subsequent infection are able to phagocytose *S. typhimurium* but, owing to their reduced oxidative burst capacity, fail to kill them, providing instead a niche for bacterial replication and dissemination.

**HO-1 is induced in immature bone marrow myeloid cells**

Given that the cytoprotective effects of HO-1, and of the heme degradation product, carbon monoxide, have been attributed to inhibition of ROS production, we wondered whether suppression of the granulocyte oxidative burst correlated with HO-1 induction during granulopoiesis. As expected, PHZ treatment and Py17XNL infection led to systemic induction of HO-1 (Supplementary Fig. 6a–c). HO-1 was consistently induced in peripheral blood monocytes by Py17XNL, PHZ and hemin, but only (to a modest extent) by hemin in circulating granulocytes and by Py17XNL in the nonmyeloid population (Supplementary Fig. 6d).

In bone marrow of untreated, uninfected mice, HO-1 is expressed mainly in F4/80+ cells (Fig. 4a), presumably macrophages and monocytes. However, in hemin-treated and PHZ-treated mice, there was a significant increase in the proportion of HO-1+ bone marrow cells, especially in the Gr-1hi/F4/80− compartment (Fig. 4a,b). There was a small but significant increase in the proportion of HO-1+ Gr-1lo/F4/80− cells in Py17XNL-infected mice (Fig. 4b) but no overall increase in bone marrow HO-1+ cells, probably owing to mobilization of F4/80+ cells from bone marrow to blood and spleen (Supplementary Fig. 7). The Gr-1lo/F4/80− compartment contains...
myeloid progenitors and immature myeloid cells. As surface markers that positively identify mouse myeloblasts and promyelocytes have not yet been defined, we assessed HO-1 expression on the monocyte/macrophage and granulocyte populations in bone marrow (GMP) using flow cytometry. We found that HO-1 expression is upregulated in the monocyte/macrophage population and downregulated in the granulocyte population upon infection with S. typhimurium. Our findings suggest that HO-1 induction in bone marrow may be a key factor in the resistance of mice to S. typhimurium infection.

**DISCUSSION**

Understanding the etiology of NTS septicemia in individuals with malaria and other hemolytic disorders may lead to new strategies for reducing morbidity and mortality. To reflect the clinical association between NTS septicemia and severe malarial anemia, we have used a model in which malaria infection causes progressive hemolysis, eventually resulting in severe (but not lethal) anemia, to assess the impact of S. typhimurium co-infection on disease. We have shown that loss of resistance to S. typhimurium requires hemolytic release of cell-free heme and subsequent induction of HO-1 and the oxidative burst in the bone marrow. However, SnPP did reverse the accumulation of granulocytes with low-level oxidative burst activity in the bone marrow of Py17XNL-infected mice (Fig. 5b), indicating that inhibition of HO restores normal development of the oxidative burst in maturing bone marrow granulocytes.

We propose (Fig. 6) that during acute hemolysis, heme triggers immediate mobilization of granulocytes from bone marrow to blood and generation of ROS, while simultaneously inducing HO-1 in immature myeloid cells and thereby reducing their subsequent oxidative burst capacity, perhaps by limiting the availability of heme for incorporation into NADPH oxidase. This results in mobilization of a heterogeneous population of granulocytes with varying levels of oxidative burst capacity. During malaria infection, however, progressive hemolysis leads to sustained release of free heme, which both impairs maturation of the oxidative burst capacity of granulocytes in the bone marrow and mobilizes functionally immature granulocytes from bone marrow into the peripheral circulation. Accumulation in peripheral blood of functionally impaired granulocytes, which phagocytose but are unable to kill bacteria, provides a new niche for bacterial infection.
replication and dissemination. In this scenario, HO-1 contributes to impaired resistance to NTS, but heme also has a direct role—either in granulocyte mobilization or as a substrate for HO-1. The heme degradation products carbon monoxide, biliverdin and iron may further impair resistance to NTS by reducing production of ROS or facilitating bacterial replication. In contrast, nonheme induction of HO-1 (for example, by CoPP) may limit available iron for bacterial replication and protect phagocytic cells from apoptosis.

Our observation that hemolysis specifically suppresses the oxidative burst capacity of neutrophils offers a plausible explanation for the particular susceptibility to NTS bacteremia in individuals with hemolysis. Salmonella species have evolved to survive and replicate inside mononuclear phagocytes; hemolysis provides an additional niche for sustained bacterial replication in circulating neutrophils. Our results are also consistent with studies of the cytoprotective role of HO-1 in mice; indeed, limitation of the granulocyte oxidative burst could be a key adaptive mechanism to reduce self-damage by ROS during hemolysis and to prevent tissue injury associated with release of heme.

Very few mechanisms have been clearly identified that confer tolerance to the harm caused by infectious organisms, despite recent interest in the therapeutic potential of augmenting host tolerance in mice, HO-1 confers tolerance to blood-stage malaria simultaneously diminishes resistance to malaria parasites developing in the liver, whereas in Drosophila, infection-induced anorexia increases tolerance against S. typhimurium but reduces resistance against Listeria monocytogenes, indicating that resistance and tolerance mechanisms can be highly pathogen specific and that a mechanism of tolerance to one pathogen can diminish resistance to another. Although it is well recognized that co-infection with different pathogens can enhance disease severity, and in some cases molecular mechanisms have been elucidated, to our knowledge, this study provides the first direct evidence in a mammalian model of tolerance to one pathogen impairing resistance to another.

To conclude, our findings have a number of key implications. First, they provide an explanation for the susceptibility to NTS bacteremia in individuals with malaria and sickle cell disease. Second, they imply...
Heme is a potent inducer of inflammation in mice and is Pyruvate kinase deficiency confers susceptibility to
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Both hemolytic anemia and malaria parasite-specific factors that tolerance and resistance mechanisms identified from studies of single pathogens may not easily translate to the ‘real world’; where people are simultaneously exposed to multiple pathogens. Specifically, the concept that the cytoprotective effects of HO-1 may be harnessed by administering its products therapeutically in humans without adversely affecting host defense against infection53,54 may not be valid. Third, we have identified a potential adjunct therapy (SnPP) that might enhance resistance to NTS in patients with hemolytic diseases. SnPP has been used experimentally to prevent severe jaundice55, but optimization of treatment would be crucial to avoid impairment of tolerance to heme. The experimental system described here may be a good starting point to assess and optimize such treatments.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

A.J.C. and J.B.d.S. conducted the experiments. A.J.C. and E.M.R. wrote the manuscript. All authors contributed to the conception and planning of the experiments, and to critical revision of the manuscript. M.W. and E.M.R. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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We determined concentrations of \( \text{Hb} \) in eight-well chamber slides and incubated at 37 °C and 5% \( \text{CO}_2 \). We used \( \text{CD11b} \) and \( \text{Gr-1} \) (eBioscience) and phycoerythrin-Cy7–conjugated antibody against \( \text{CD11b} \) by flow cytometry (staining with allophycocyanin-conjugated antibody against \( \text{CD11b} \) and killing of \( \text{Salmonella} \) phagocytosis and killing assays.

We assessed cells with overlapping GFP before nuclear staining and mounting with DAPI dissolved in confocal matrix. For light microscopy, we fixed thin blood films with methanol and Giemsa-stained thin blood smears. We determined erythrocyte counts using a Z2 Coulter particle counter. We induced acute hemolysis by subcutaneous treatment or 8 h before \( \text{Hb} \) measurement.

We assessed neutrophil oxidative burst and degranulation by the percentage increase in the median fluorescence intensity of surface \( \text{CD11b} \) expression in stimulated versus unstimulated samples.

We determined total hemoglobin using QuantiChrom Heme and Hemoglobin assay kits (BioAssay Systems). We quantified protein-bound plasma \( \text{Hb} \) using a previously described spectrophotometric method and the concentration of plasma hemoglobin using a previously published method.

**Hmox1 expression and HO activity assays.** We determined \( \text{Hmox1} \) mRNA expression in liver by quantitative RT-PCR. We standardized cDNA expression for each sample using the housekeeping genes \( \text{Gapdh} \) and \( \text{Tbp} \) and calculated expression as relative fold change compared to healthy control samples. We measured HO activity in whole-liver homogenates after red blood cell lysis using a previously described method. We measured plasma HO-1 by ELISA using an HO-1 Immunoset (Enzo Life Sciences).

**Statistical analyses.** We performed statistical analysis using Graph Pad Prism 5 software. We used an alpha value of 0.05 for all preplanned statistical analyses. We compared proportions between groups using Fisher's exact test. We used the log-rank Mantel Cox test for survival analysis. We analyzed continuous data that were approximately normally distributed using two-sided unpaired or paired Student's \( t \)-test for pairwise comparisons or one-way ANOVA with post hoc testing using Dunnett's multiple comparison test for comparison with the control group, Tukey's multiple comparison test for comparison between multiple groups and Bonferroni's multiple comparison test for comparison between two or more selected pairs. We log\(_{10}\)-transformed all data relating to bacterial loads before analysis. We compared proportions between groups using Fisher's exact test.

**Additional methods.** Detailed methodology is described in the Supplementary Methods.

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Erratum: Malaria impairs resistance to *Salmonella* through heme- and heme oxygenase–dependent dysfunctional granulocyte mobilization

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In the version of this article initially published, the received date was incorrect. The correct date is 25 July 2011. The error has been corrected in the HTML and PDF versions of the article.