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

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Abstract

In sub-Saharan Africa, invasive non-Typhoid Salmonella (NTS) 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 might 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 S. typhimurium causes acute, fatal bacteremia with increased bacterial load; features reproduced by phenylhydrazine 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[1] and NTS co-infection has been associated with increased malaria mortality[2]. The association of NTS infection with hemolysis is well established in humans with malaria (especially severe malarial anemia)[3-4] and sickle cell disease[5], and in mice with hemolysis due to rodent malaria infection[6-8], treatment with phenylhydrazine or anti-erythrocyte antibodies, or red blood cell enzyme defects [9-11]. It has been assumed that hemolysis-induced macrophage dysfunction was 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[7-8,10].

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

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Hemolysis results in liberation of heme, leading to expression of the inducible isoform of heme oxygenase-1 (HO-1)[12] which degrades heme to biliverdin, carbon monoxide and iron[13]. Heme is pro-oxidant, induces neutrophil migration and activates the neutrophil oxidative burst[14-16] but HO-1 (and its products) play an essential cytoprotective role (reviewed in [17]) - dramatically demonstrated by the severe susceptibility to oxidative stress in mice and humans with HO-1 deficiency[18-20]. HO-1 induction has been shown to protect against infectious, inflammatory and hypoxic-ischemic insults in mice (reviewed in [21]) and has been linked to modulation of malarial pathogenesis[22] and sickle cell disease[23]. Recently, in mice, induction of HO-1 has been proposed as a tolerance mechanism in severe malaria[24-26] and polymicrobial sepsis[27]: HO-1 reduces heme-mediated tissue damage and enhances survival without reducing pathogen load. An important 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 [17]). However, ROS are important for resistance to certain pathogens, including Salmonella[28], and this raises the intriguing 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 cause impaired resistance to S. typhimurium bacteremia

To determine whether heme liberated by hemolysis impairs resistance to NTS infection, we compared survival and bacterial loads following intraperitoneal infection with green fluorescent protein (GFP)-expressing *S. enterica* serovar Typhimurium 12023 (hereafter referred to as *S. typhimurium*) in C57BL/6 mice with or without preceding *Plasmodium yoelii* 17XNL (Py17XNL) co-infection, phenylhydrazine (PHZ) or hemin treatment. Py17XNL infection of C57BL/6 mice causes a self-resolving infection; parasitemia peaks at 20-30% and is accompanied by progressive hemolytic anemia (Fig. 1a). By contrast, PHZ treatment causes acute hemolysis (Fig. 1b). In both cases, plasma heme concentrations are markedly increased and similar to concentrations achieved 12 hours after injection of hemin (Fig. 1c), but without depletion of haptoglobin or hemopexin (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.

Decreased survival of malaria-infected, PHZ- or hemin-treated mice after *S. typhimurium* infection was accompanied by increased bacterial loads in whole blood, spleen, liver and bone marrow (Fig. 1e) and bacteremia was much more pronounced: immediately before death (i.e. 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).

Salmonella localize in granulocytes following hemolysis and hemin treatment

By flow cytometry, we identified GFP^+ (Salmonella-containing) cells in blood, spleen and bone marrow. In the blood of Py17NXL-infected mice, and of PHZ- or hemin-treated mice, just before death, Salmonellae were found predominantly in $Gr\text{-}1^{Hi}$ cells (Fig. 2a), and were enriched in this cell population compared to control (PBS-treated) mice (Fig. 2b). The proportion of all $Gr\text{-}1^{Hi}$ cells in blood, spleen and bone marrow which were GFP^+ (Fig. 2c)

correlated with the bacterial load determined by culture (Fig. 1e,f). The Gr-1^{Hi} cells were identified as granulocytes (Ly6G^{+} F4/80^{-}CD115^{-}; Supplementary Fig. 2a,b). Almost all GFP^{+} cells were CD115^{-} (Fig. 2d); moreover, Salmonella infection caused an increase in the proportion of Gr-1^{Lo}CD115^{-} blood leukocytes (Fig. 2e), suggesting that immature granulocytes are mobilized from the bone marrow to the peripheral blood during infection[29]. In support of this, blood films from Py17XNL-infected and PHZ or hemin-treated mice 18 h after Salmonella infection (Fig. 2f) revealed numerous neutrophils containing *S. typhimurium* and many of these neutrophils had immature nuclear morphology. In contrast, neutrophils from PBS-treated mice displayed mature nuclear morphology and did not contain Salmonella.

The accumulation of GFP^{+} bacteria in granulocytes was not simply due to failure of bacterial uptake by monocytes and macrophages, since the proportions of GFP^{+} cells in the spleen which were either monocytes or macrophages (F4/80^{Lo}CD11b^{Hi} and F4/80^{Hi}CD11b^{Lo} 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 Salmonella killing

Since there is no obvious defect in uptake of *S. typhimurium* by macrophages and monocytes following 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 rates of phagocytosis of *S. typhimurium* between neutrophils or monocytes or between cells from malaria-infected or uninfected mice (Fig. 3a,b); the intracellular location of GFP^{+} bacteria was confirmed by confocal microscopy (Supplementary Fig. 3a,b). However, when cells were lysed after 2 h in the gentamicin protection assay and live bacteria enumerated by culture, live bacterial recovery from cells from Py17XNL-infected mice was significantly higher than from control mice (Fig. 3b) indicating significant impairment of intracellular killing of *S. typhimurium* by cells from Py17XNL-infected mice.

Since HO-1 reduces the production of ROS[30-33], and since phagocyte NADPH oxidase is essential for early resistance to *S. typhimurium*[28], we investigated whether Py17XNL infection impaired the granulocyte oxidative burst. Using oxidation of dihydrorhodamine to its fluorescent derivative rhodamine to assess oxidative burst[34], we observed progressive suppression of the PMA-induced oxidative burst of blood granulocytes during infection (Fig. 3c). Granulocytes were not simply refractory to PMA stimulation since PMA-induced degranulation (assessed by surface CD11b expression[35]) was actually enhanced 14 and 21 days after malaria infection (Fig. 3d). However, blood granulocytes isolated 24 h after PHZ or hemin treatment did not differ from those of control mice in oxidative burst capacity, degranulation, *ex vivo* phagocytosis or killing of *S. typhimurium* (Fig. 3c,d and Supplementary Fig. 3c,d).

Hemolysis induces dysfunctional granulocyte mobilization

Accumulation of heme following PHZ-hemolysis or hemin administration is faster than during Py17XNL infection. Heme directly induces neutrophil migration and ROS production[16] whilst subsequent HO-1 induction in myeloid cells suppresses neutrophil maturation and ROS production[30,33]. Since HO-1 is induced in bone marrow by hemolysis[26], we wondered whether the chronic hemolysis associated with Py17XNL infection might induce HO-1 in immature bone marrow myeloid cells, suppress their

oxidative burst capacity as they mature, and allow gradual accumulation of dysfunctional cells in the circulation, whereas acute hemolysis (induced by PHZ) 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[29]. Gr-1^{Hi} cells are mature neutrophils and Gr-1^{Lo} cells are immature granulocytes and granulocyte progenitors; the Gr-1^{Int} (intermediate) compartment contains a mixture of cell types. Generation of an oxidative burst is restricted to a functionally mature subpopulation of cells[36] in the Gr-1^{Hi}, and to a lesser extent Gr-1^{Int}, compartment (Supplementary Fig. 4).

Hemin and PHZ treatment, and Py17XNL infection, all caused striking depletion of Gr-1^{Hi} cells from bone marrow (Fig. 3e and Supplementary Fig. 5). For PHZ and hemin treatment, loss of Gr-1^{Hi} 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 marow to the periphery. Although the proportion of circulating granulocytes did not increase during Py17XNL infection, granulocyte mobilization may be obscured by an overall increase in leukocyte count or granulocyte redistribution (eg. from blood to the spleen)[37]. *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, the oxidative burst of circulating granulocytes was assessed. Eight hours after *S. typhimurium* infection the oxidative burst response to PMA was enhanced in PBS-treated mice (presumably due to priming[38]) but oxidative burst capacity was dramatically reduced in PHZ treated mice (Fig. 3f).

Finally, we investigated whether maturation of the oxidative burst in bone marrow granulocytes was also impaired, as predicted. 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-1^{Hi} cells (Fig. 3g), evident as an increase in the proportion of cells with low oxidative burst capacity (Fig. 3h) and a decrease in the proportion of cells with high burst capacity, 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, due to 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

Since the cytoprotective effects of HO-1, and of the heme degradation product, carbon monoxide, have been attributed to inhibition of ROS production[17,39], we wondered whether suppression of granulocyte oxidative burst correlated with HO-1 induction during granulopoiesis. As expected[25,40], 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 non-myeloid 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 and PHZ-treated mice there was a significant increase in the proportion of HO-1^{+} bone marrow cells, especially in the Gr-1$^{Lo/-}$F4/80^{-} compartment (Fig. 4a,b). There was a small but significant increase in the proportion of HO-1^{+} Gr-1^{Lo} F4/80^{-} cells in Py17XNL-infected mice (Fig. 4b) but no overall increase in bone marrow HO-1^{+} cells, likely due to mobilization of F4/80^{+} cells from bone marrow to blood and spleen[37,41] (Supplementary Fig. 7). The Gr-1$^{lo/-}$F4/80^{-} compartment contains myeloid progenitors and immature myeloid cells. Since surface markers to positively identify murine myeloblasts and promyelocytes have not yet been defined, we assessed HO-1 expression in the granulocyte macrophage progenitor (GMP) population which is proximal to the myeloblast in the myeloid differentiation pathway[42]. Py17XNL, PHZ and hemin all caused significant induction of HO-1 even at this very early stage of development (Fig. 4c). Thus, malaria infection, hemolysis and hemin treatment all induce HO-1 expression in the earliest stages of granulocyte development and thereby impair subsequent functional maturation of these cells.

Impaired resistance to Salmonella is abrogated by HO inhibition

To test the hypothesis that HO-1 impairs resistance of Py17XNL-infected mice to *S. typhimurium* bacteremia, we pre-treated mice with the competitive HO inhibitor, tin protoporphyrin IX (SnPP). Treatment of Py17XNL-infected mice with SnPP for 48 h prior to *S. typhimurium* infection reduced bacterial loads in blood, spleen and liver to levels not significantly different from those in PBS treated mice (Fig. 5a). SnPP treatment had no effect on bacterial load in mice without Py17XNL infection (Fig. 5a), or on parasitemia in Py17XNL-infected mice (Fig. 5b) but very effectively prevented accumulation of GFP^{+} *S. typhimurium* within granulocytes in Py17XNL-infected mice (Fig. 5c,d). SnPP also partially restored resistance to *S. typhimurium* when administered 2 h prior to PHZ treatment (Fig. 5a,c,d) and prolonged the survival of Py17XNL or PHZ-treated mice after *S. typhimurium* infection (Fig. 5e). Cobalt protoporphyrin (CoPP), which induces HO-1 in the absence of hemolysis or free heme[43], did not impair resistance to *S. typhimurium* 16 h post infection (Fig. 5a). Thus, both heme and HO-1 are necessary for impairment of resistance to *S. typhimurium* caused by Py17XNL or PHZ hemolysis, and inhibition of HO abrogates this effect. Inhibition of HO by SnPP did not restore the oxidative burst of Gr-1^{Hi} bone marrow granulocytes in Py17XNL-infected or PHZ-treated mice (Fig. 5f), presumably due to enhanced mobilization of mature Gr-1^{Hi} cells (Fig. 5g) as a result of greater heme accumulation (Fig. 3e, Supplementary Fig. 5). However, SnPP did reverse the accumulation of granulocytes with low level oxidative burst activity in the bone marrow of Py17XNL-infected and PHZ-treated mice (Fig. 5h), indicating that inhibition of HO restores normal development of the oxidative burst in maturing bone marrow granulocytes.

Discussion

Understanding the etiology of NTS septicemia in individuals with malaria and other hemolytic disorders may lead to new strategies to reduce morbidity and mortality. To reflect the clinical association between NTS septicemia and severe malarial anemia[3-4] we have used a model in which malaria infection causes progressive hemolysis, eventually resulting in severe (but non-lethal) anemia, to assess the impact of *S. typhimurium* co-infection on disease. We demonstrate that loss of resistance to *S. typhimurium* requires hemolytic release of cell-free heme and subsequent induction of HO-1, and that inhibition of HO-1 reverses this susceptibility to NTS. Thus, although HO-1 is essential for tolerance to the cytotoxic effects of free heme, reducing disease severity without altering pathogen load[24-27], HO-1-mediated tolerance to malaria simultaneously impairs resistance to *S. typhimurium*.

We propose (Fig 6) that during acute hemolysis, heme triggers immediate mobilisation of granulocytes from bone marrow to blood and generation of ROS[16], whilst simultaneously inducing HO-1 in immature myeloid cells and thereby reducing their subsequent oxidative burst capacity[30,33], perhaps by limiting the availability of heme for incorporation into NADPH oxidase[44]. 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 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 replication and dissemination. In this scenario, HO-1 contributes to impaired resistance to NTS but heme also plays a direct role – either in granulocyte mobilization[16] 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[21] or facilitating bacterial replication[45]. In contrast, non-heme induction of HO-1 (e.g. by CoPP) may limit available iron for bacterial replication and protect phagocytic cells from apoptosis[45-46].

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 have evolved to survive and replicate inside mononuclear phagocytes[47]; 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 an important adaptive mechanism to reduce self-damage by ROS during hemolysis and to prevent tissue injury associated with release of heme.

Very few tolerance mechanisms have been clearly identified[48], despite recent interest in their therapeutic potential [49]. In mice, HO-1 confers tolerance to blood stage malaria[24-25] but simultaneously diminishes resistance to malaria parasites developing the liver[50]. However, in Drosophila, infection-induced anorexia increases tolerance against *S. typhimurium* but reduces resistance against *Listeria monocytogenes* [51], 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[52], this study provides the first direct evidence in a mammal of tolerance to one pathogen impairing resistance to another.

To conclude, our findings have a number of important implications. First, they provide an explanation for the susceptibility to NTS bacteremia in malaria and sickle cell disease patients. Second, they imply 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 defence against infection[53-54] may not be valid. Third, we have identified a potential adjunct therapy (SnPP) which might enhance resistance to NTS in patients with hemolytic diseases. SnPP has been used experimentally to prevent severe jaundice[55] 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

Methods

Detailed methods are provided as Supplementary Methods.

We stored aliquots from a single broth culture of *Salmonella enterica* serovar Typhimurium 12023 (*S. typhimurium*), constitutively expressing GFP, in 10% glycerol at −80 °C. For *in vitro* experiments we opsonized Salmonella in 20% normal mouse serum at 37°C for 30 min prior to inoculation. For quantitation of bacterial loads, we plated 10 fold-dilutions of lysed organ homogenates and whole blood onto LB agar and incubated for 18 hours before counting the number of colony forming units (CFUs).

Animals

Animal experimentation conformed with UK Home Office Regulations and was approved by Institutional ethical review (London School of Hygiene and Tropical Medicine). We obtained female, 6-10 week old C57BL/6 mice from Harlan and Charles River, UK, and infected them with *Plasmodium yoelii* 17X non-Lethal (Py17XNL) by i.p. injection of 10^5 parasitised red blood cells (pRBCs). We determined parasitemia by examination of Giemsa-stained thin blood smears. We determined erythrocyte counts using a Z2 Coulter particle counter. We induced acute hemolysis by subcutaneous injection of phenylhydrazine (125 μg g^{-1}). We administered hemin by i.p. injection (50 μmol kg^{-1} per dose) in two doses 12 hours apart. We initiated Salmonella infections by i.p. inoculation of 10^5 CFU of *S. typhimurium* on day 15 of Py17XNL infection or 6 h after PHZ or first dose of hemin treatment. We administered SnPP (40 μmol kg^{-1} per dose) by i.p. injection 48, 24 and 8 h before *S. typhimurium* infection of Py17XNL infected mice, 2 h before PHZ treatment, or 8 h before *S. typhimurium* alone. We administered CoPP (10mg kg^{-1} body weight) i.p. 6 h before *S. typhimurium* infection. We monitored *S. typhimurium* infected mice 6 hourly until they displayed signs of illness (clinical stage 2, see Supplementary Methods) and then every 1-2 h until they reached the humane endpoint (clinical stage 4), at which point they were euthanized. The time of euthanasia was used for survival analysis.

Flow cytometry

Antibodies used are shown in Supplementary Methods. We performed intracellular staining for HO-1 based on the method of Ewing *et al*[56].

Microscopy

Chamber slides were protected from light and air-dried for 2 h before nuclear staining and mounting with DAPI dissolved in confocal matrix. To determine whether bacteria were intracellular or adherent to the cell surface, we assessed cells with overlapping GFP^+ *S.*

typhimurium by 0.5 μm interval Z-stack imaging. For light microscopy, we fixed thin blood films with methanol and air dried before staining with May-Grünwald Giemsa stain).

Salmonella phagocytosis and killing assays

We assessed *ex-vivo* phagocytosis and killing of *S. typhimurium* in a gentamicin protection assay and quantified by flow cytometry (staining with APC-conjugated antibody against Gr-1 and PE-Cy7-conjugated antibody against CD11b), confocal microscopy and bacterial culture. We seeded isolated $CD11b^+$ cells at 1×10^5 per well in flat bottomed 96-well plates or at 2×10^5 per well in 8-well chamber slides and incubated at 37°C and 5% CO_2 for 20 min prior to addition of *S. typhimurium* at a multiplicity of infection (MOI) of 10 *S. typhimurium*: 1 $CD11b^+$ cell. We determined phagocytosis at 45 min after infection and killing at 2 h after infection.

Oxidative burst and degranulation assay

We assessed neutrophil oxidative burst using a modification of the flow cytometric assay described by Richardson et al.[34] in which dihydrorhodamine 123 is converted to rhodamine. We measured neutrophil degranulation by the percentage increase in the median fluorescence intensity of surface CD11b expression in stimulated versus unstimulated samples.

Measurement of heme and hemoglobin

We determined concentrations of hemin and hemoglobin using Quantichrom Heme and Hemoglobin assay kits. We quantified protein bound plasma heme using the spectrophotometric method of Shinowara and Waters[57] and the concentration of plasma hemoglobin using the method of Khan *et al*.[58].

Hmox1 expression and HO activity assays

We determined *Hmox1* mRNA expression in liver by quantitative RT-PCR. We standardized cDNA expression for each sample using the housekeeping genes *Gapdh* and *Tbp* and calculated expression as relative fold change compared to healthy control samples. We measured HO activity in whole liver homogenates after RBC lysis, using the method of Foresti *et al.* [59]. We measured plasma HO-1 by ELISA using a HO-1 Immunoset.

Statistical analysis

We performed statistical analysis using Graph Pad Prism 5 software. We used an alpha value of 0.05 for all pre-planned statistical analyses. We used the Log Rank Mantel Cox test for survival analysis. We analyzed continuous data which 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 prior to analysis. We compared proportions between groups using Fisher's exact test.

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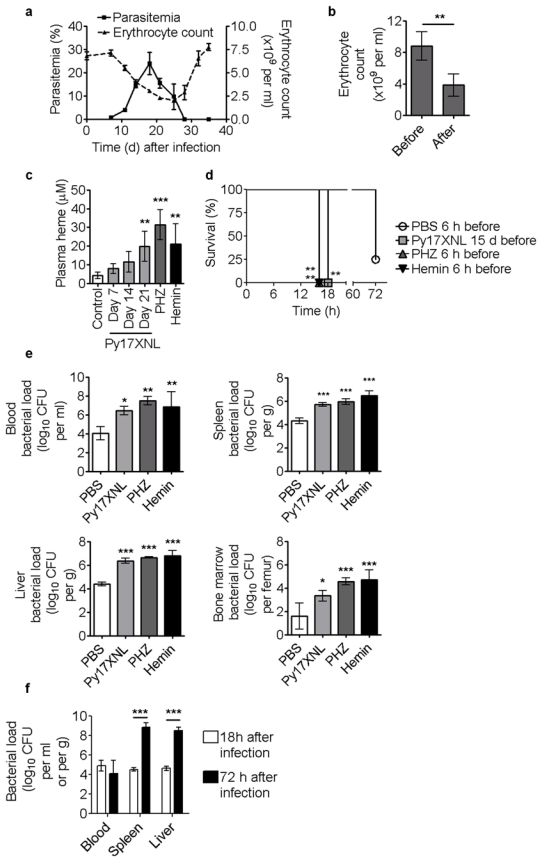

Figure 1. Hemolysis and heme are associated with impaired resistance to *S. typhimurium*

(**a**) Erythrocyte count and parasitemia of mice infected with *Plasmodium yoelii* 17XNL (Py17XNL). Data representative of 7 independent experiments (mean ± s.d. of 5-25 mice per time point). (**b**) Erythrocyte count before- and 18 h after - subcutaneous injection of phenylhydrazine (PHZ). Data representative of 3 independent experiments (mean ± s.d. of 5 mice). (**c**) Plasma heme levels during Py17XNL infection and 15 h after PHZ- or 12 h after hemin treatment. Data representative of at least 2 independent experiments (mean ± s.d. of 4-5 mice) per condition and time point. (**d**) Survival (time until reaching humane endpoint) of mice infected with *S. typhimurium* on day 15 of Py17XNL or 6 h after PHZ, first dose of hemin or PBS treatment. Data representative of ≥4 independent experiments (mean ± s.d. of 4-5 mice) per condition. (**e**) *S. typhimurium* bacterial loads in whole blood, spleen, liver, and bone marrow, 18 h post-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 end-point) post-infection for PBS-treated mice. (**e,f**). Data representative of ≥2 independent experiments (mean ± s.d. of 4-5 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**). $*P < 0.05$,$**P < 0.01$, $***P < 0.001$.

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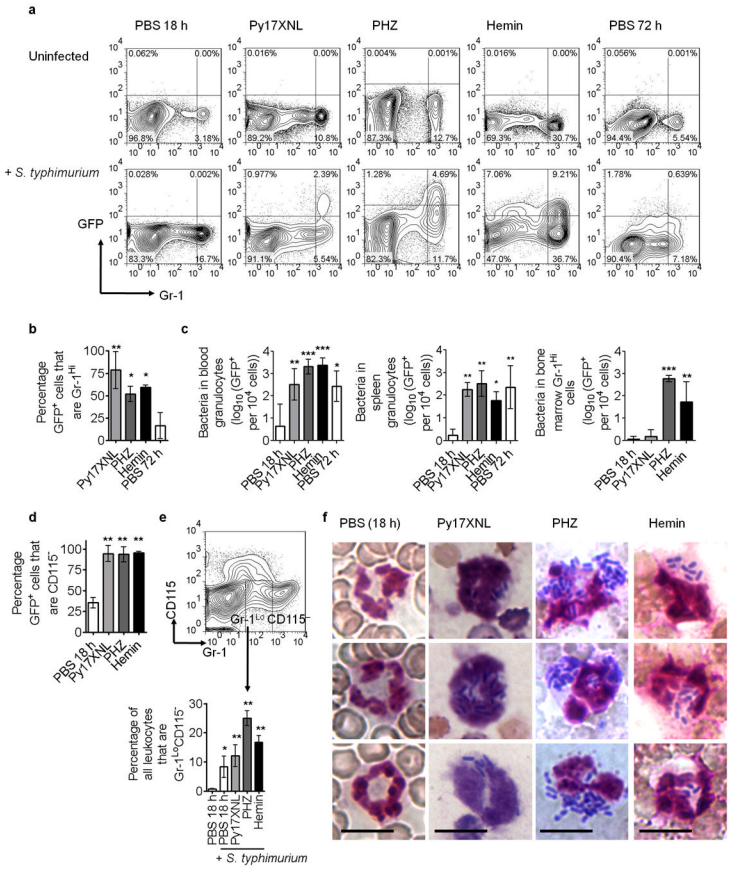

Figure 2. *S. typhimurium* localize in granulocytes following hemolysis and hemin treatment

(**a**) Representative flow cytometry plots of blood leukocytes collected from GFP-*S. typhimurium*- infected (lower panels) and uninfected (upper panels) mice at the humane endpoint or indicated timepoint. Data representative of ≥4 independent experiments (n = 4-5 mice) per condition. (**b**) Proportion of all GFP^+ blood leukocytes with high levels of Gr-1 expression (PBS 18 h not shown due to very low absolute numbers of GFP^+ cells). (**c**) Infected mature granulocytes ($GFP^+Gr\text{-}1^{Hi}$) as a proportion of all $Gr\text{-}1^{Hi}$ cells in blood, spleen and bone marrow. (**d**) Proportion of all GFP^+ cells in blood that are $CD115^-$. (**b-d**) Data representative of ≥2 independent experiments (mean ± s.d. of 4-5 mice) per condition. (**e**) Representative flow cytometry analysis defining immature granulocytes as $Gr\text{-}1^{Lo}CD115^-$ (upper panel) and quantification of the proportion of blood leukocytes that are $Gr\text{-}1^{Lo}CD115^-$ (lower panel). Data combined from 2 independent experiments (mean ± s.d. of 4-9 mice) per condition. (**f**) Light microscope images of neutrophils from May-Grünwald Giemsa-stained thin blood films of *S. typhimurium* infected mice, 18 h after infection in PBS treated mice, or at the humane endpoint (Py17XNL infected and PHZ or hemin treated mice). Images are representative of neutrophils containing bacteria, except for PBS treated mice where no bacteria were seen, from 2 independent experiments with 3-5 mice per condition. Scale bar 10 μm. Significance determined by one-way ANOVA with Dunnett's multiple comparison test. $*P < 0.05$,$**P < 0.01$, $***P < 0.001$.

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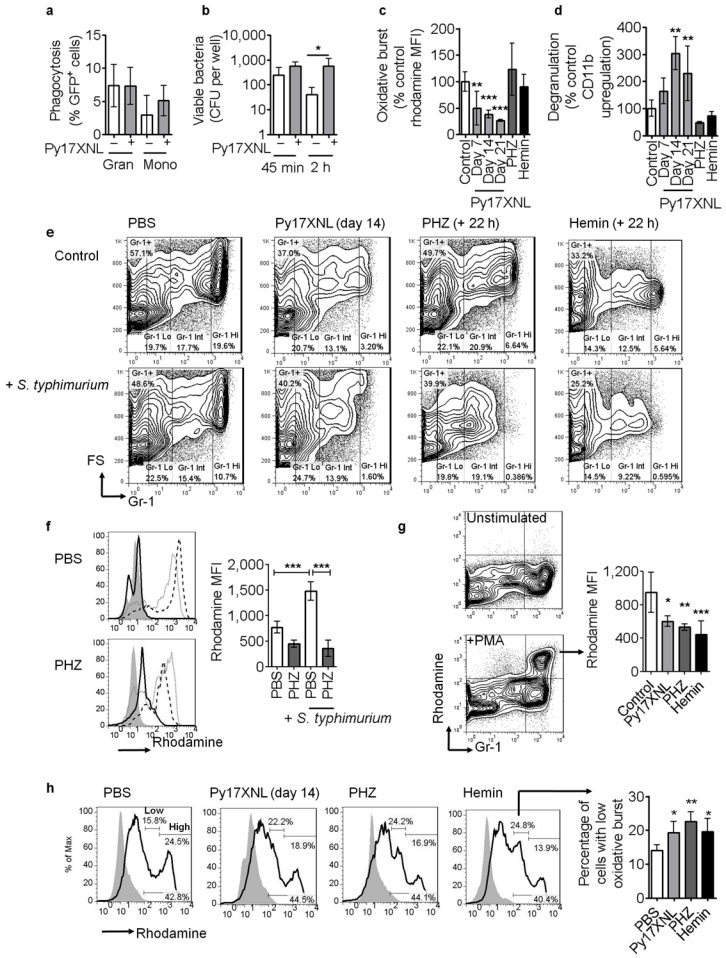

Figure 3. Hemolysis and heme cause dysfunctional granulocyte mobilization

In vitro phagocytosis (**a,b**) and killing (**b**) of *S. typhimurium* by CD11b^{+} cells from blood of control and Py17XNL infected (day 15) mice. (**a**) Phagocytosis: percentage of granulocytes (Gran) and monocytes (Mono) which were GFP^{+}. (**b**) Phagocytosis (45 min) and killing (2 h) of *S. typhimurium* assessed by quantitative culture. Data representative of 2 independent experiments (mean ± s.d. of 3-5 mice per condition). (**c,d**) PMA-stimulated oxidative burst (**c**) and degranulation (**d**) of granulocytes in whole blood from control, Py17XNL-infected and PHZ- or hemin-treated (24 h after first treatment) mice, relative to mean of PBS controls. Representative of ≥2 independent experiments (mean ± s.d. of 3-8 mice) per condition and timepoint. (**e**) Flow cytometry analysis of Gr-1 expression by bone marrow cells in uninfected mice (upper panels) or 16 h after *S. typhimurium* infection (lower panels). Representative of ≥4 independent experiments per condition. (**f**) PMA-stimulated oxidative burst of whole blood granulocytes 14 h after PHZ or PBS treatment (6 h after *S. typhimurium* infection). Rhodamine fluorescence for unstimulated-uninfected (grey filled), unstimulated-infected (heavy line), PMA-stimulated-uninfected (light line) and PMA-stimulated-infected (dashed line), PBS- and PHZ-treated mice (left panels); quantitative data for PMA-stimulated blood (right panel). Representative of 2 independent experiments (mean ± s.d. of 4 mice per condition). (**g**) PMA-stimulated oxidative burst of Gr-1 ^{Hi} bone marrow granulocytes from control, day 14 Py17XNL, and hemin and PHZ treated mice. Representative of ≥3 independent experiments (mean ± s.d. of 3-5 mice) per condition. (**h**) Proportion of Gr-1^{Hi} bone marrow cells making low level oxidative burst response to PMA. Total, low and high level oxidative burst populations (left panels; unstimulated control in

grey) and quantitative data (right panel). Representative of ≥3 independent experiments (mean ± s.d. of 3-5 mice) per condition. Significance determined by two-tailed Student's *t*-test (**a,b,f**) or one-way ANOVA with post-hoc comparison with control using Dunnett's multiple comparison test (**c,d,f,g,h**). $*P < 0.05$,$**P < 0.01$, $***P < 0.001$.

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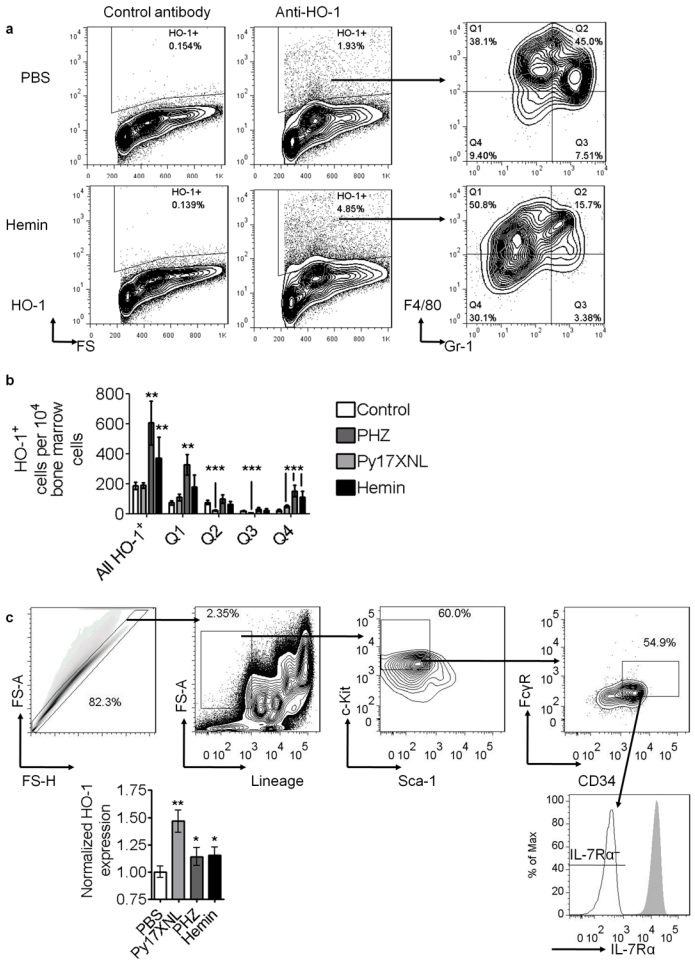

Figure 4. HO-1 induction in bone marrow

(**a**) Representative flow cytometry plots identifying bone marrow cells expressing HO-1, which were then divided based on expression of F4/80 and Gr-1. (**b**) Quantification of the proportion of bone marrow cells expressing HO-1, overall and in each quadrant (Q1-4) based on F4/80 and Gr-1 expression in (**a**), for control PBS-treated, Py17XNL-infected (day 14), PHZ- and hemin-treated mice (18 h after treatment). Data representative of 2 independent experiments (mean ± s.d of 3-5 mice) per condition. (**c**) Flow cytometry analysis showing identification of granulocyte macrophage progenitor (GMP) cells as lineage^{-} c-Kit^{+} Sca-1^{-} FcγR^{+}CD34^{+}IL-7Ra^{-}, and normalized HO-1 expression in GMP cells determined by the ratio of the MFI of the fluorescence for the antibody against HO-1 to control antibody staining for the same sample, normalized against the average expression in PBS-treated mice in the same experiment (lower left panel). The IL7-Ra^{+} population (shaded, lower right plot) is drawn from the Lineage^{Hi} population in the upper, second left plot. Data from 2 independent experiments, (mean ± s.d.) 4-8 mice per condition. Significance was determined by one-way ANOVA with post-hoc comparison with control condition using Dunnett's multiple comparison test. *$P < 0.05$,**$P < 0.01$, ***$P < 0.001$.

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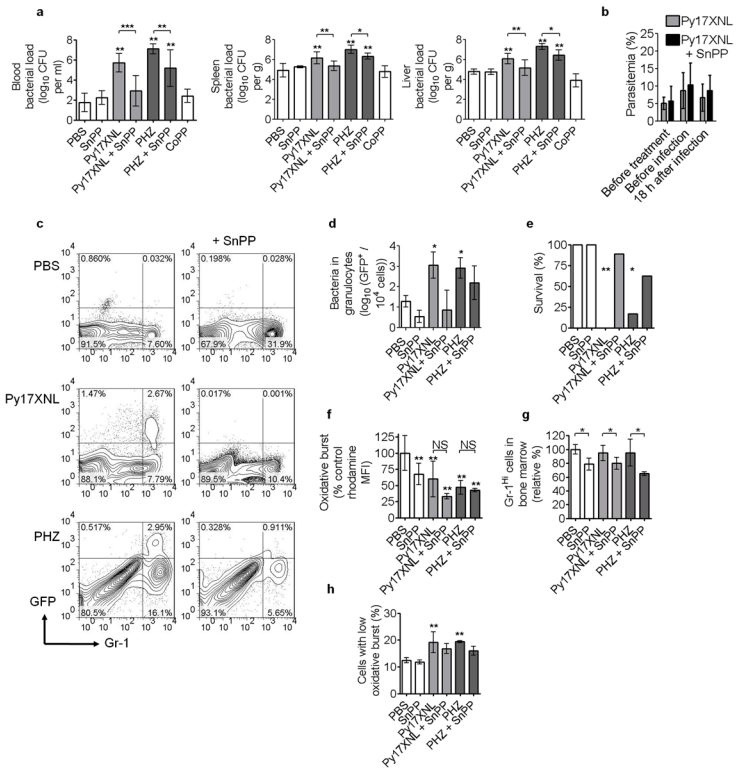

Figure 5. Impaired resistance to *S. typhimurium* is abrogated by inhbition of heme oxygenase

(**a**) *S. typhimurium* bacterial loads in blood, spleen and liver, of mice treated and harvested as in Fig. 1e, with additional groups receiving SnPP or CoPP treatment. (**b**) Parasitemia of SnPP-treated and untreated Py17XNL-infected mice immediately before treatment with SnPP, immediately before infection with *S. typhimurium*, and 18 h after infection. (**c**) Representative flow cytometry analysis of blood leukocytes from *S. typhimurium* -infected mice treated as in **Fig. 5a**. (**d**) Quantification of GFP^+ granulocytes as a proportion of all granulocytes in blood. (**e**) Survival (to the humane endpoint) 18 h or 16 h after *S. typhimurium* infection in Py17XNL-infected and PHZ-treated mice respectively, with or without SnPP treatment. (**a-e**) Data represent pooled results (mean ± s.d) of 2 independent experiments (6-9 mice per condition). (**f**) The oxidative burst of $Gr\text{-}1^{Hi}$ bone marrow granulocytes (as in Fig. 3g). (**g**) Proportion of bone marrow cells that are $Gr\text{-}1^{Hi}$ following SnPP treatment, expressed as a percentage of the average proportion of $Gr\text{-}1^{Hi}$ cells in the equivalent treatment condition without SnPP. (**h**) Proportion of $Gr\text{-}1^{Hi}$ cells responding to PMA with a low-level oxidative burst (as in Fig. 3h). Data representative of 2 independent experiments (mean ± s.d of 3-5 mice) per condition. Significance determined by one-way ANOVA with post-hoc comparisons using Dunnett's multiple comparison test or Bonferroni's multiple comparison (bracketed groups) (**a,b,d,f,h**), Student's t-test (**g**) and Fisher's exact test (**e**). $*P < 0.05$,$**P < 0.01$, $***P < 0.001$.

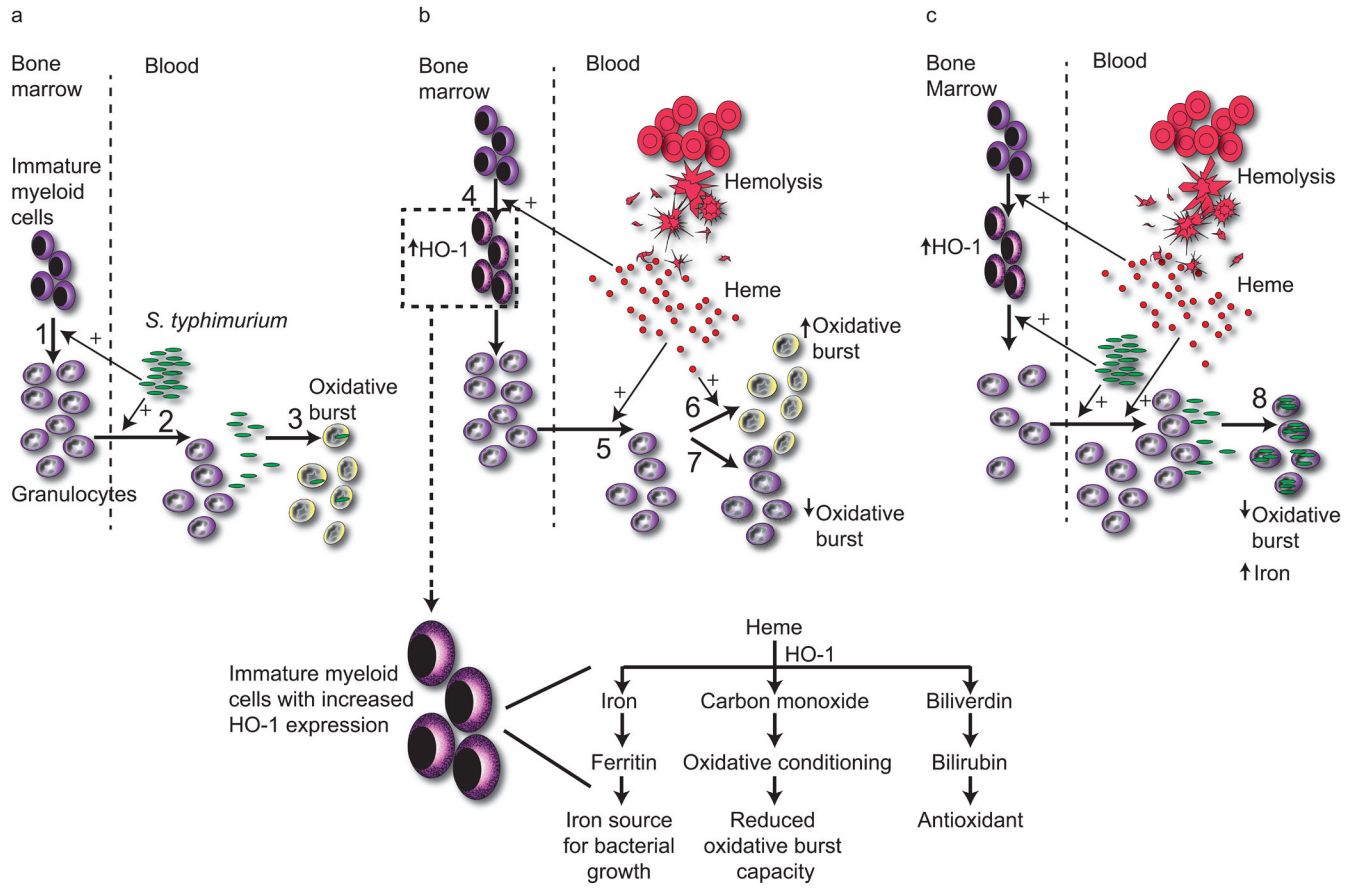

Figure 6. Proposed mechanism to explain how hemolysis impairs resistance to *S. typhimurium* through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization

Proposed scheme for (**a**) granulocyte mobilization in response to *S. typhimurium* infection and (**b**) the dysfunctional mobilization induced by hemolysis which causes (**c**) impaired resistance to *S. typhimurium*. In normal C57BL/6 mice (**a**), *S. typhimurium* infection causes emergency granulopoiesis (**1**) and mobilization of granulocytes from bone marrow (**2**). These granulocytes are able to phagocytose *S. typhimurium* and generate a normal oxidative burst, controlling bacterial replication (**3**). Intravascular hemolysis caused by malaria or phenyhydrazine treatment liberates hemoglobin and hemoglobin-derived heme (**b**). Heme induces heme oxygenase-1 (HO-1) in immature myeloid cells (**4**), and heme is degraded to biliverdin, carbon monoxide and iron (**magnified box**). These heme degradation products may modify the function of developing granulocytes. Heme also causes mobilization of mature granulocytes from bone marrow (**5**), and may activate the oxidative burst (**6**). However, in chronic hemolysis (as in the case of Py17XNL infection), functionally immature granulocytes accumulate in the blood, ultimately resulting in reduced oxidative burst capacity (**7**). The combination of hemolysis and *S. typhimurium* challenge (**c**), increases mobilization of bone marrow granulocytes with reduced oxidative burst capacity and increased cellular iron stores into the blood. These granulocytes are able to phagocytose *S. typhimurium* normally, but are unable to kill the bacteria due to reduced oxidative burst capacity, and support bacterial growth due increased iron availability, ultimately allowing increased bacterial replication and dissemination (**8**).

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