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

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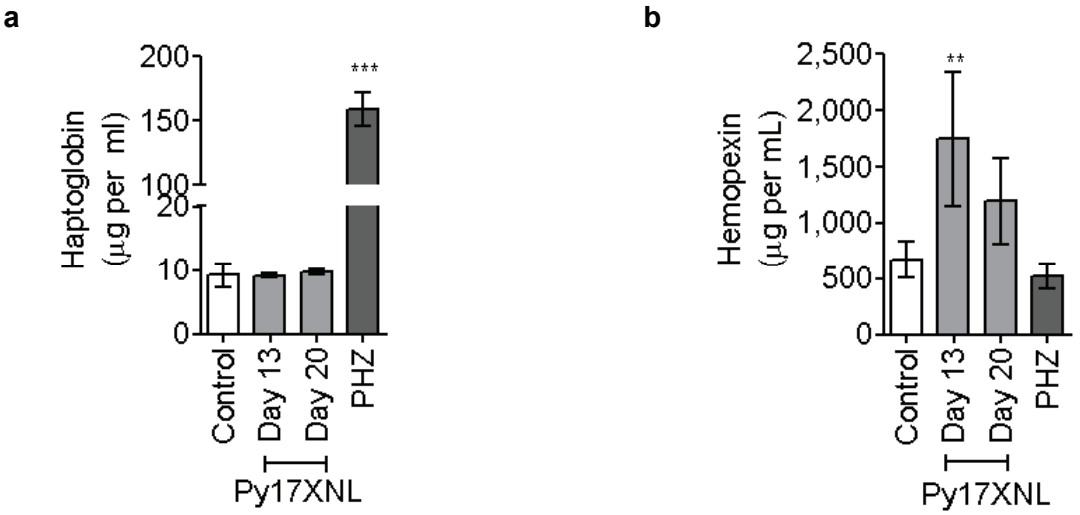
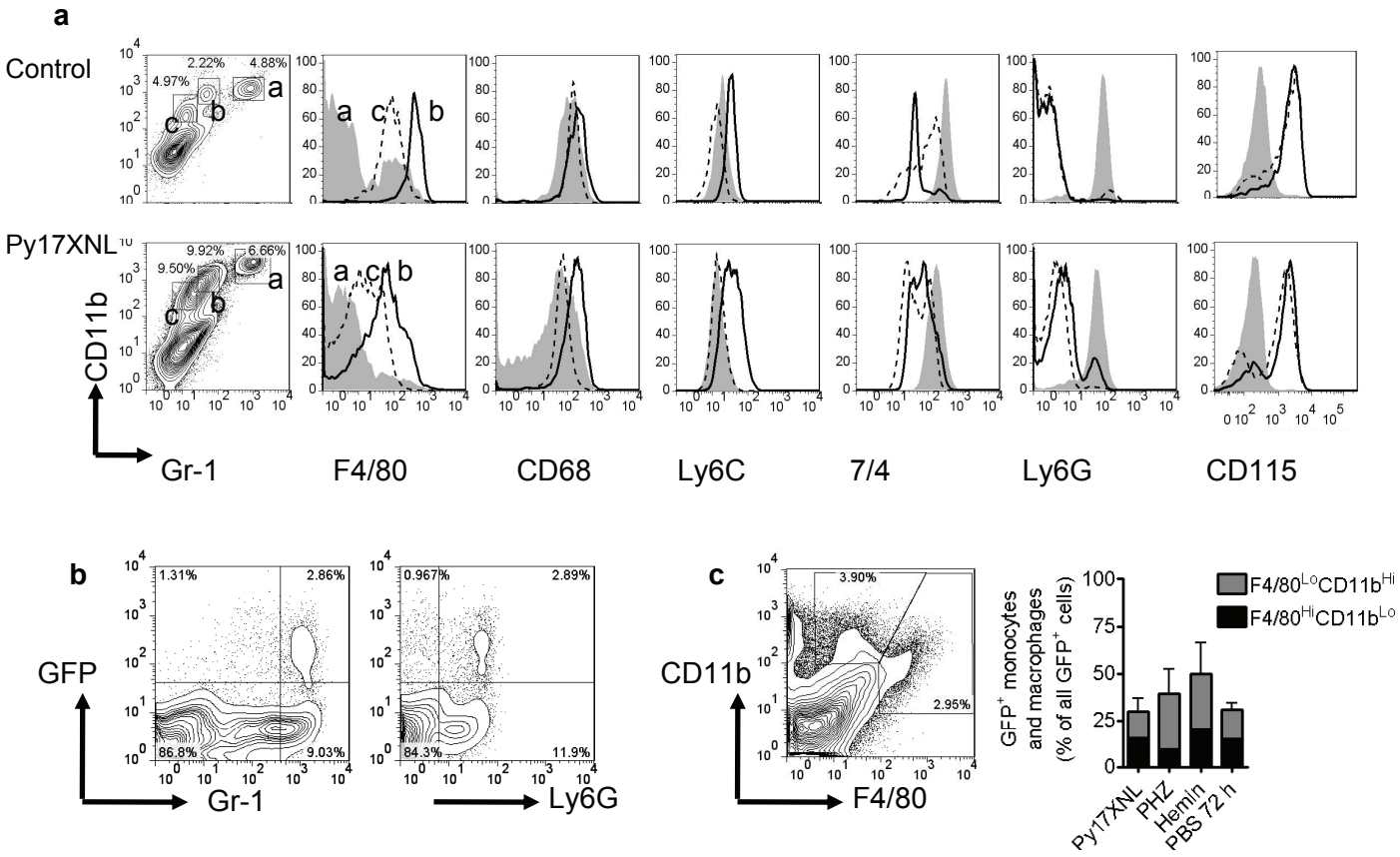
Supplementary Figure 1



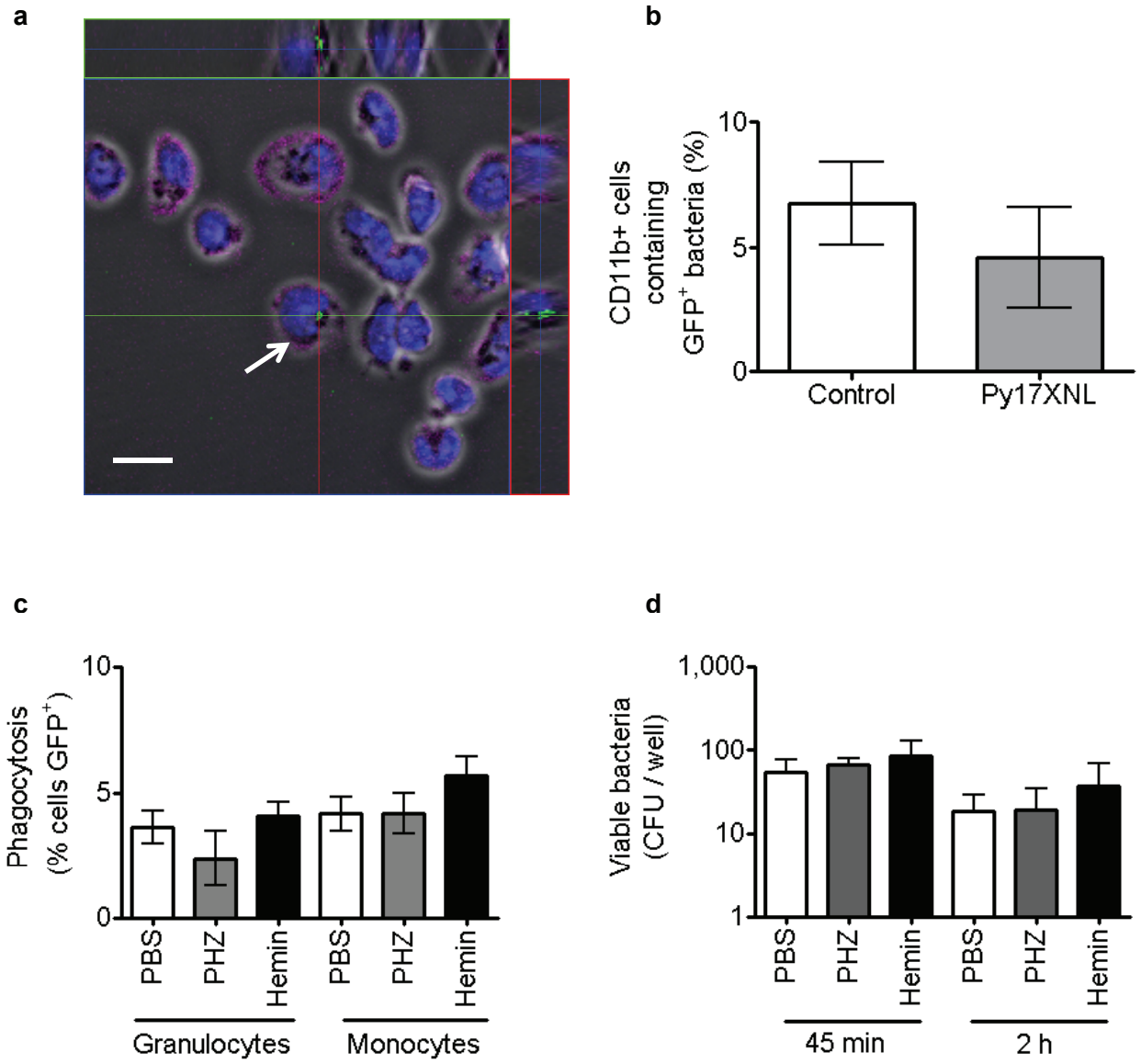
Supplementary Figure 1. Plasma haptoglobin and hemopexin levels are not depleted by Py17XNL or phenylhydrazine hemolysis. (**a,b**) Plasma haptoglobin (**a**) and hemopexin (**b**) were measured by ELISA during Py17XNL infection and 18 h after phenylhydrazine (PHZ) administration. Data are representative of 2 independent experiments (mean ± s.d. of 3–4 mice) per condition. Significance was determined by one-way ANOVA with post-hoc comparison with control condition using Dunnett's multiple comparison test. ***P* <0.01, ****P* <0.001.

Supplementary Figure 2



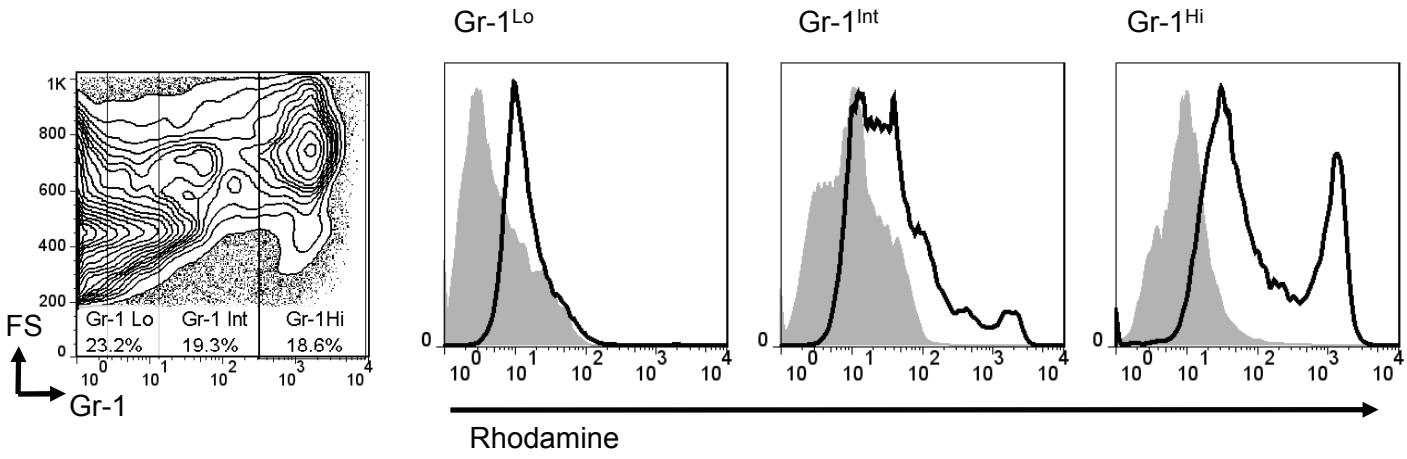
Supplementary Figure 2. Definition of myeloid cell populations by flow cytometry. (**a**) Myeloid cell populations in blood were defined by differential expression of CD11b and Gr-1 in both uninfected and Py17XNL-infected mice (14 d post infection), and their identity confirmed by expression of other surface markers (histograms). Regions a, b, c (and filled, solid line and dashed line histograms) correspond to granulocyte, inflammatory monocyte and resident monocyte populations respectively. (**b**) Representative flow cytometric analysis of blood 18 h after infection with GFP-expressing *S. typhimurium* on day 15 of Py17XNL infection. Granulocytes were identified by Gr-1^{Hi} expression (left hand panel) and Ly6G expression (right hand panel).(**c**) Splenic monocyte and macrophage populations were defined by flow cytometry as F4/80^{Lo}CD11b^{Hi} and F4/80^{Hi}CD11b^{Lo} respectively (left hand panel) and the proportion of all GFP^{+} positive cells in each of these compartments was quantified in Py17XNL-infected, PHZ- and hemin-treated mice at the humane endpoint (right hand panel). GFP^{+} cells are shown 72 h after infection of PBS-treated control mice, but due to very low infection levels in PBS-treated mice 18 h after infection, resulting in no GFP^{+} cells being detected in most mice, this condition is not shown. Data are representative of 2 independent experiments with 3–5 mice per condition. Significance was determined by one-way ANOVA with post-hoc comparison with control condition (PBS) using Dunnett's multiple comparison test.

Supplementary Figure 3.



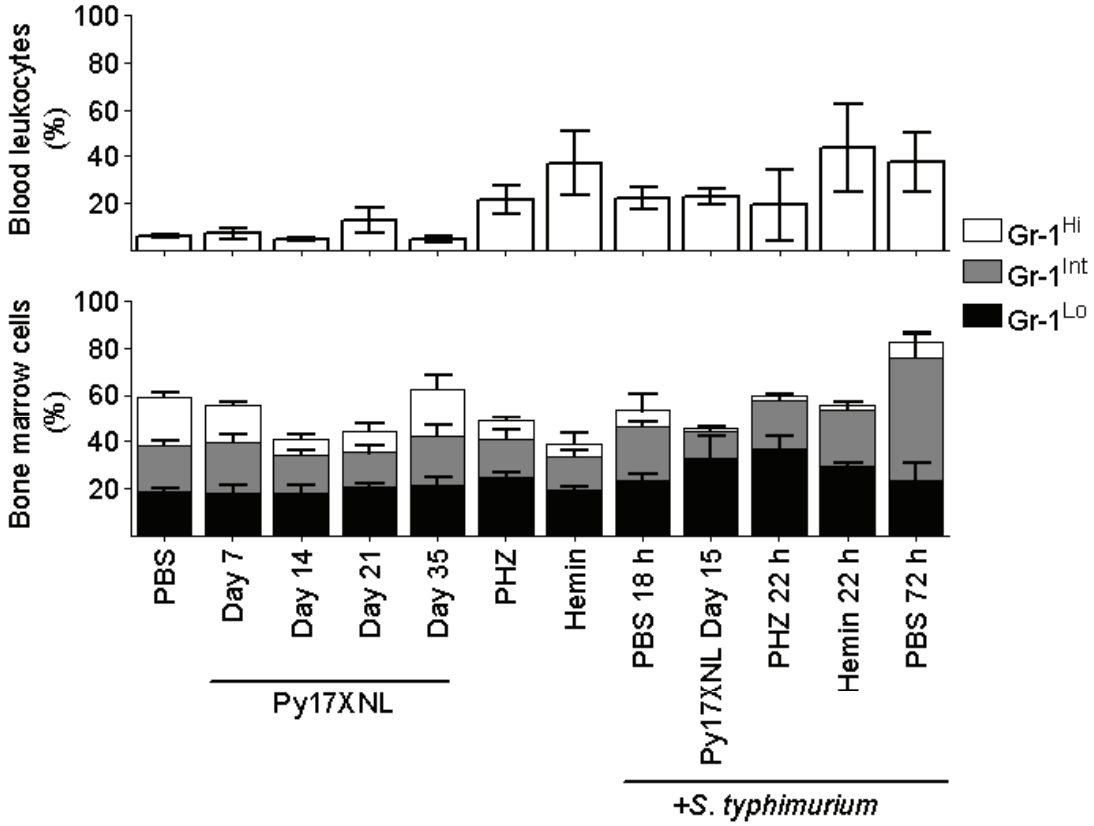
Supplementary Figure 3. Phagocytosis of *S. typhimurium* by monocytes and neutrophils is not impaired by Py17XNL, phenylhydrazine or hemin and *S. typhimurium* killing is not impaired by phenylhydrazine or hemin. (**a,b**) Intracellular bacteria were quantified by confocal microscopy following incubation of CD11b$^+$ cells (isolated from blood of Py17XNL-infected (day 14) and uninfected control mice) with *S. typhimurium* for 45 min in a gentamicin protection assay. (**a**) Representative orthogonal reconstruction from 17 stacked images at 0.5 µm depth intervals, showing a CD11b$^+$ cell (from Py17XNL infected mouse, arrow) containing GFP$^+$ *S. typhimurium*. Blue nuclear staining, DAPI; Green, GFP; scale bar 10 µm. (**b**) Summary data for analysis of confocal images. Data are representative of 2 independent experiments (mean ± s.d. of 3 mice) per condition. Phagocytosis (**c,d**) and killing (**d**) of *S. typhimurium* by CD11b$^+$ cells isolated from whole blood of control mice or test mice 24 h after PHZ or hemin treatment, and incubated with *S. typhimurium in vitro* in a gentamicin protection assay. Phagocytosis was assessed by the percentage of granulocytes and monocytes which were GFP$^+$ by flow cytometry **(c)** and by culture of cell lysates collected after 45 min incubation with bacteria (**d**). Bacterial killing was assessed by culture of cell lysates collected after 2 h incubation with bacteria (**d**). Data are representative of 2 independent experiments (mean ± s.d. of 3–6 mice) per condition. Significance was determined by two-tailed Student's *t*-test (**b**) or one-way ANOVA with post-hoc comparison with control condition using Dunnett's multiple comparison test (**c,d**).

Supplementary Figure 4



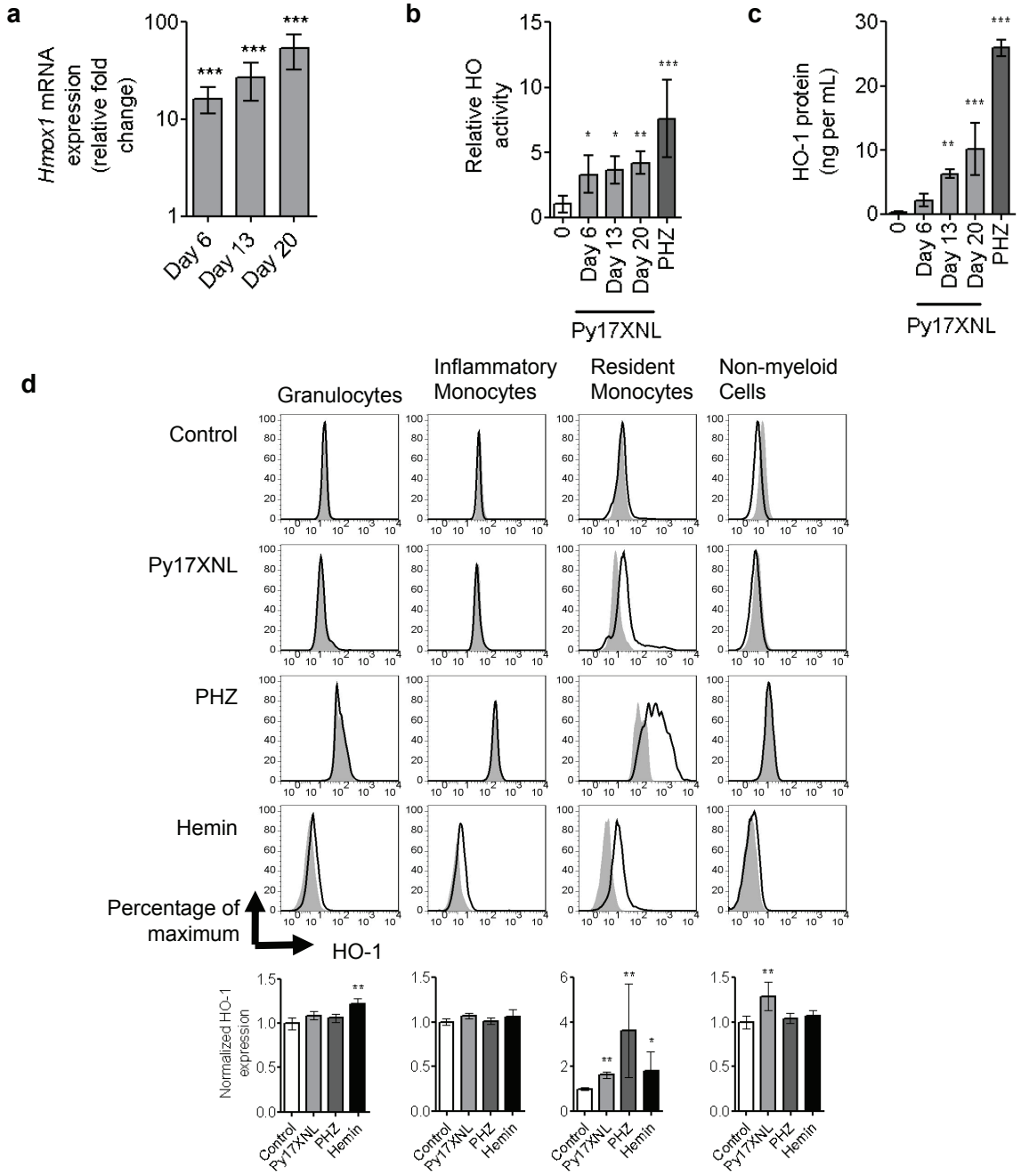
Supplementary Figure 4. Oxidative burst activity is predominantly a property of Gr-1^{Hi} bone marrow granulocytes. Left hand panel: Representative flow cytometric analysis of control bone marrow cells showing gating of Gr-1^{Lo}, Gr-1^{Int} (Gr-1 intermediate) and Gr-1^{Hi} cells. Right hand panels: rhodamine fluorescence intensity of unstimulated (shaded) and PMA-stimulated cells (unshaded, black line). Results are representative of 6 experiments each with 3–5 mice.

Supplementary Figure 5



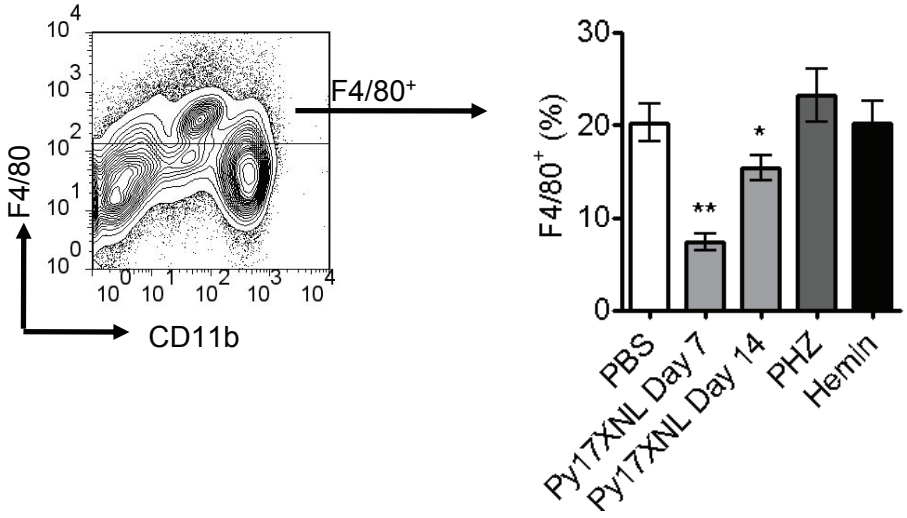
Supplementary Figure 5. Frequency and phenotype of bone marrow and peripheral blood granulocytes. Flow cytometric analysis of blood (upper panel) and bone marrow (lower panel) granulocytes assessed by expression of Gr-1 and divided as low, intermediate and high expression ($Gr\text{-}1^{Lo}$, $Gr\text{-}1^{Int}$, $Gr\text{-}1^{Hi}$ respectively), 22 h after mice were treated with PBS, PHZ or hemin treatments, or at different time points during Py17XNL infection, or with additional *S. typhimurium* infection 16 h (PHZ and hemin), 18 h (Py17XNL and PBS) or 72 h (PBS) before harvest. Granulocyte frequencies are shown as a percentage of all leukocytes in blood, and of all bone marrow cells, respectively. Data are representative of at least 3 independent experiments (mean ± 95% confidence interval of 3–5 mice) per condition and time point.

Supplementary Figure 6



Supplementary Figure 6. Hemolysis and hemin cause systemic and cell-type specific induction of HO-1. Heme oxygenase-1 induction assessed by *Hmox1* mRNA expression in liver (**a**), HO bio-activity in liver (**b**) and HO-1 protein concentration in plasma (**c**). (**a**) *Hmox1* expression in liver was determined by rt-PCR expressed as fold change relative to the control gene *Gapdh*, relative to the difference between *Hmox1* and *Gapdh* expression in control liver. Data are representative of 2 independent experiments (mean ± s.d. of 4–5 mice per time point). Similar results were obtained using *Tbp* as the control gene. (**b**) HO enzyme activity in liver homogenates from mice infected with Py17XNL or 18 h after PHZ treatment, was determined by conversion of hemin to bilirubin, standardized for protein content and expressed relative to controls at each time point. Data are representative of 2 independent experiments (mean ± s.d. of 3–11 mice per condition and time point). (**c**) Plasma HO-1 protein concentration was determined by ELISA. Data are representative of 2 independent experiments (mean ± s.d. of 4 mice per condition or time point). (**d**) HO-1 expression in myeloid and non-myeloid cells in blood was determined by flow cytometry, using the cell population definitions shown in **Supplementary Fig 2a**. HO-1 expression was quantified (lower panels) by the ratio of anti-HO-1 fluorescence (unfilled histograms) relative to control antibody (filled histograms) (upper panels) and normalized to the average value for control animals in each experiment. Data are representative of at least 2 independent experiments with 3–9 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.

Supplementary Figure 7



Supplementary Figure 7. Frequency of F4/80$^+$ cells in bone marrow. Flow cytometric analysis of the proportion of bone marrow cells staining positively for the monocyte/ macrophage marker F4/80, during Py17XNL infection or 22 h after PBS, PHZ and hemin treatment. Data are representative of 1 or 2 independent experiments (mean ± s.d. of 3–11 mice) per condition and time point. 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.

Supplementary methods

Reagents. All reagents were purchased from Sigma unless specified otherwise. Hemin (ferriprotoporphyrin IX chloride), tin protoporphyrin IX (SnPP), and cobalt (III) protoporphyrin IX chloride (CoPP) were obtained from Frontier Scientific, were protected from light and prepared by dissolving in 0.2 M NaOH, diluted to the desired concentration in PBS and buffered carefully to pH 7.5 with HCl. Hemin solutions were subsequently filtered through a 0.2 µm acrodisc syringe filter unit (Pall Corporation) and the concentration of the filtered solution determined using a Quantichrom Heme assay (BioAssay Systems) according to the manufacturer's instructions. SnPP and CoPP solutions were not filtered but were prepared using sterile reagents and technique. Aliquots of hemin (3.26 mg ml^{-1}), SnPP (3 g ml^{-1}) and CoPP (1mg ml^{-1}) were stored at –80 ^{0}C until use. Phenylhydrazine hydrochloride 25 mg ml^{-1} solution was freshly prepared immediately prior to injection by dissolving in PBS, buffering to pH 7.4 with NaOH, and filtering through a 0.2 µm syringe unit.

Salmonella enterica serovar Typhimurium 12023 (*S. typhimurium*) constitutively expressing GFP was a gift from Prof. David Holden (Imperial College, London, UK). Bacteria were grown to late log phase in static culture in Luria-Bertani (LB) broth with 50 µg ml^{-1} carbenicillin. Bacteria were frozen in aliquots in 10% Glycerol and stored at –80 ^{0}C until required for use. The concentration of stock bacteria was determined by dilution cultures on LB Agar, and reconfirmed for each aliquot at the time of use. Prior to inoculation, the Salmonella stock was defrosted, washed twice in PBS and diluted to the desired concentration in sterile PBS. For *in vitro* experiments Salmonella were opsonized in 20% normal mouse serum at 37^{0}C for 30 min prior to inoculation. For quantitation of bacterial loads from organs of Salmonella infected mice, cell suspensions were prepared by disruption of tissue with a syringe plunger, passage through a 70 µM nylon cell strainer (BD), and resuspension as 10% solution by weight in sterile PBS. 10 fold-dilutions of organ homogenates and whole blood were made in 1% Triton X-100, plated onto LB agar and incubated for 18 h 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. Female, 6–10 week old C57BL/6 mice were obtained from Harlan and Charles River, UK and maintained under barrier conditions. Frozen stocks of blood-stage *Plasmodium yoelii* 17X Non-Lethal (Py17XNL) were inoculated in passage mice. Blood was collected after 5-7 days and experimental animals were infected by intraperitoneal (i.p.) injection of 10^5 parasitised red blood cells (pRBCs). Parasitemia was determined by examination of Giemsa-stained thin blood smears. Erythrocyte count was determined using a Z2 Coulter particle counter (Beckman Coulter). Parasitemia, erythrocyte count and, where relevant, body weight were monitored at least least twice a week. To induce acute hemolysis, mice were treated with phenylhydrazine (125 µg g^{-1} body weight) by subcutaneous (s.c.) injection. Hemin was administered by i.p. injection (50 µmol kg^{-1} body weight per dose) in two doses 12 h apart. Salmonella infections were initiated by i.p. inoculation of 10^5 CFU of *S. typhimurium* in 200 µL PBS, on day 15 of Py17XNL infection or 6 h after PHZ or first dose of hemin treatment. To inhibit heme oxygenase activity, SnPP (40 µmol kg^{-1} $dose^{-1}$) was administered 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. The HO-1 inducer CoPP (10mg kg^{-1}) was administered i.p. 6 h prior to *S. typhimurium* infection. Control animals received injections of equivalent volumes of PBS. After infection with *S. typhimurium*, mice were monitored 6 hourly until displaying signs of illness (clinical stage 2, **Supplementary Methods Table 1**) and then every 1-2 h until they reached clinical stage 4, at which point they were euthanized. Since progression from stage 4 to death is extremely rapid in Salmonella and rodent malaria infections, and using death as an experimental endpoint was considered unethical, the humane endpoint - time of reaching stage 4 - was used for survival analysis. In each experiment a group of PBS-treated Salmonella-infected mice was sacrificed at the same time as the treatment groups to allow comparison of bacterial loads. In all experiments with groups of Salmonella-infected mice, animals were killed by injection of pentobarbitol. In all other experiments mice were killed with CO_2 inhalation. Immediately after death, under aseptic technique, blood was collected by cardiac puncture into heparinised syringes and tissues were removed into ice cold RPMI and stored on ice, protected from light, until processing.

Table 1. Clinical scale used to determine disease severity in mice:

1. no signs
2. ruffled fur and/or abnormal posture and/or minor weight loss (<15%)
3. lethargy and/or moderate weight loss (≥ 20%)
4. reduced response to stimulation and/or ataxia and/or respiratory distress/hyperventilation
5. prostration and/or paralysis and/or convulsions and/or severe weight loss (>25%)
6. Death

The humane endpoint was defined as mice reaching stage 4

Flow cytometry. Antibodies used are shown in **Supplementary Methods Table 2**. For all experiments except oxidative burst assays, cells were incubated for 5 min with red blood cell lysing buffer (Sigma), washed, and resuspended in FACS buffer prior to staining. Cells from *S. typhimurium* infected mice were fixed in 4% formaldehyde prior to surface staining. Cells were incubated with cocktails of antibodies for 30 min at room temperature in the dark and washed twice before analysis.

Intracellular staining for HO-1 was based on the method of Ewing *et al* [56]. Briefly, cells were fixed in 2% formaldehyde for 10 min at 37^0C, centrifuged at 500 g for 5 min at 4^0C, resuspended in ice-cold 90% methanol and incubated on ice for an additional 30 min. After washing with FACS buffer, cells were resuspended in FACS buffer containing 1% normal mouse serum (Southern Biotech) and Mouse Fc Block (BD Biosciences) or PE conjugated antibody against CD16/32 and incubated for 5 or 30 minutes at room temperature respectively. Cells were then centrifuged at 1000g for 2 minutes and resuspended in FACS buffer containing rabbit polyclonal antibody against HO-1 or an equivalent concentration of normal rabbit polyclonal antibody as a control for 30 min at 4^0C. Cells were washed twice in FACS buffer before resuspension with FITC conjugated secondary antibody and a cocktail of surface marker antibodies and incubation at 4^0C for 30 min, or room temperature for 90 min when the antibody against CD34 was used, followed by 2 final washes.

A BD FACSCalibur flow cytometer was used to acquire all samples except those for analysis of bone marrow progenitor populations (which were acquired using a BD LSR-II) and data were analysed using FlowJo version 7.6 (Tree Star, Inc.).

Microscopy. Chamber slides were protected from light and air-dried for 2 h before nuclear staining and mounting with DAPI dissolved in confocal matrix (Micro-Tech-Lab). Slides were examined using a Zeiss axiovert confocal microscope with a Plan-Apochromat 63x oil immersion lens and Zeiss LSM510 analysis software. For quantitative assessment of phagocytosis, cells with overlapping GFP^+ *S. typhimurium* were assessed further by 0.5 µm interval Z-stack imaging to determine whether bacteria were intracellular or adherent to the cell surface. For light microscopy, thin blood films were fixed with methanol and air dried before staining with May-Grünwald Giemsa stain according to the manufacturer's instructions. Images were acquired using a Zeiss Axioplan2 microscope with CP Apochromat 100x oil immersion lens, and images were obtained with a Retiga 2000R camera (QImaging) and analysed using Volocity 5.5.1 software (PerkinElmer).

Salmonella phagocytosis and killing assays. Ex-vivo phagocytosis and killing of *S. typhimurium* by blood granulocytes and monocytes were assessed in a gentamicin protection assay and quantified by flow cytometry, confocal microscopy and bacterial culture. Following red blood cell lysis, $CD11b^+$ cells were isolated from murine whole blood using anti-CD11b magnetic beads (Miltenyi), according to the manufacturers' instructions. After washing twice in DMEM (Gibco), cells were resuspended at $5.6x10^5$ ml^{-1} in DMEM without antibiotics and seeded at $1x10^5$ per well in flat bottomed 96-well plates or at $2x10^5$ per well in 8-well chamber slides (Labtek). Plates and slides were incubated at 37 ^{0}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. To control for autofluorescence and binding of opsonised *S. typhimurium* to the cell surface without phagocytosis, wells containing uninfected cells (negative control), or wells in which both cells and bacteria were fixed with 2% formaldehyde (fixed controls), were incubated in parallel. Plates were incubated for 15 min before addition of gentamicin to a final concentration of 100 µg ml^{-1} to kill remaining extracellular bacteria (ie. those which had not been phagocytosed) and incubation for another 30 min. To assess bacterial phagocytosis, after 30 min cells were washed twice with warm sterile PBS and either harvested for flow cytometry, fixed *in situ* for confocal microscopy, or lysed for bacterial culture. Alternatively, to assess bacterial killing, cells were washed twice with warm medium, then reincubated for 2 h in medium containing 10 µg ml^{-1} gentamicin to prevent extracellular growth of *S. typhimurium*. To assess phagocytosis by flow cytometry, cells were gently scraped from wells (on ice) and resuspended in PBS with 2% formaldehyde before staining with APC-conjugated antibody against Gr-1 and PE-Cy7-conjugated antibody against CD11b. Phagocytosis was quantified as the proportion of GFP^+ cells after subtraction of the proportion of GFP^+ cells in the fixed control samples. To assess phagocytosis and bacterial survival by culture, cells were washed twice in warm sterile PBS to remove gentamicin, then lysed with 1% Triton X-100 and 10-fold dilutions were plated onto LB agar, incubated for 18 h at 37^0C and colonies counted. To assess phagocytosis by confocal microscopy, cells were fixed with 2% formaldehyde for 15 min at room temperature, then washed twice in PBS containing 5% fetal calf serum, and allowed to air dry before staining and mounting.

Oxidative burst and degranulation assay. Neutrophil oxidative burst was assessed using a modification of the flow cytometric assay described by Richardson *et al*.[34]. 50µL aliquots of fresh whole blood, or bone marrow suspension (cells from 1 femur resuspended in 500 µl RPMI), were mixed with either 50 µL of PMA solution (stimulated samples, 25 µM for whole blood, 2.5 µM for bone marrow) or sterile PBS (unstimulated) and incubated for 15 minutes in a 37^0C water bath. Next 25 µL of a staining cocktail containing dihydrorhodamine 123 and fluorochrome- conjugated antibodies to cell surface markers in PBS was added and cells reincubated for 5 min at 37^0C. 2 ml of red blood cell lysis buffer was added to each tube and incubated in the dark for 15 min at room temperature then centrifuged at 1000 g for 1 min. Cells were washed again with PBS, and resuspended in PBS with 1% paraformaldehyde. The magnitude of the oxidative burst was assessed by rhodamine median fluorescence intensity (MFI) measured in the FL-1 channel, analysed on the same day. Degranulation was measured by the percentage increase in the MFI of surface CD11b expression in stimulated versus unstimulated samples. In each experiment at least three control samples (from age-matched, healthy, uninfected mice) were assayed in parallel with experimental samples. For longitudinal assessment of the oxidative burst and degranulation at different time points in the same experiment, rhodamine fluorescence values were normalized to the average value for the control samples at each time point.

Measurement of heme and hemoglobin. Standard hemoglobin solution was prepared by 0.2 µm filtration of lysed red blood cell supernatant. The concentrations of standard solutions of hemin and hemoglobin were determined using Quantichrom Heme and Hemoglobin assay kits (BioAssay Systems) in accordance with the manufacturer's instructions. Protein bound plasma heme was quantified by the spectrophotometric method of Shinowara and Waters[57] using a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies). Briefly heparinised whole blood was centrifuged at 500g for 5 minutes, then plasma was removed and subjected to centrifugation at 15000g for a further 7 minutes to pellet any remaining red blood cells. Two µL of the remaining plasma was used to determine absorbance at 562, 578, 598, 615 and 675nm wavelengths. Using the absorption values at 562, 578 and 598nm the concentration of plasma hemoglobin was determined by the method of Khan *et al.*[58]. The effect of plasma hemoglobin on the difference in absorption 615–675 nm was determined by preparation of a standard curve of hemoglobin in plasma, and the absorption difference 615–675nm was corrected for the effect of plasma hemoglobin. The corrected absorption at 615–675nm was used to determine plasma heme from a standard curve for hemin in plasma.

Hmox1 expression, HO activity and HO-1 protein assays. *Hmox1* mRNA expression in liver was determined from fresh snips of liver which were snap frozen in liquid nitrogen and stored at -80^0C until processing. RNA was extracted (RNAeasy, Qiagen) and DNAse1- treated prior to cDNA synthesis. cDNA was quantified using pre-validated inventoried Taqman gene expression assays for *Hmox1* (Mm00516004_m1), *Gapdh* (Mm99999915_g1) and *Tbp* (Mm00446973_m1), and an ABI Prism 7000 sequence detection system (Applied Biosystems). cDNA expression for each sample was standardized using the housekeeping genes *Gapdh* and *Tbp* and expressed as relative fold change compared to healthy control samples. HO activity was measured in whole liver homogenates after RBC lysis, using the method of Foresti *et al.*[59]. To allow comparison between experiments, HO activity was normalized to the average value of at least three control samples in each experiment. Plasma HO-1 was measured by ELISA using a HO-1 Immunoset (Assay Designs) performed in accordance with the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using Graph Pad Prism 5 software. All statistical analyses were pre-planned and used an alpha value of 0.05. Survival analysis was performed using the Log Rank Mantel Cox test. Continuous data which were approximately normally distributed were analysed 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. All data relating to bacterial loads were $\log_{10}$-transformed prior to analysis. Comparison of proportions between groups was performed using Fisher's exact test.

Supplementary Methods Table 2: antibodies used for flow cytometry

Antigen (clone)	Manufacturer	Fluorochrome
Gr-1 (RB6-8C5)	eBioscience	FITC, PE, PE-Cy7, APC, efluor 450
CD11b (M1/70)	eBioscience	PE, PE-Cy7
Ly6G (1A8)	Miltenyi Biotec	APC
F4/80 (BM8)	eBioscience	FITC, APC
CD68 (FA-11)	AbDSerotec	AlexaFluor 647
Ly6C (Hk1.4)	Abcam Biolegend	FITC APC
Ly6B.2 (7/4)	AbDSerotec	FITC
CD115 (AFS98)	eBioscience	PE, APC
CD34 (RAM34)	eBioscience	eFluor 660
CD16/32 (93)	eBioscience	PE
CD127 / IL-7Rα (A7R34)	eBioscience	PERCP-Cy5.5
Sca-1 / Ly6A/E (D7)	eBioscience	PE-Cy7
c-Kit / CD117 (ACK2)	eBioscience	APC-eFluor 780
Mouse haematopoietic lineage cocktail	eBioscience	eFluor 450
HO-1 (SPA-895, polyclonal rabbit)	Assay Designs	None, primary antibody
Polyclonal rabbit serum	Covance	None, primary antibody
F(ab')2 Anti-Rabbit IgG (secondary antibody)	eBioscience	FITC, secondary antibody