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Prolonged Neutrophil Dysfunction after \textit{Plasmodium falciparum} Malaria Is Related to Hemolysis and Heme Oxygenase-1 Induction

Aubrey J. Cunnington,* Madi Njie,† Simon Correa,† Ebako N. Taken,† Eleanor M. Riley,* and Michael Walther†,‡

It is not known why people are more susceptible to bacterial infections such as nontyphoid \textit{Salmonella} during and after a malaria infection, but in mice, malarial hemolysis impairs resistance to nontyphoid \textit{Salmonella} by impairing the neutrophil oxidative burst. This acquired neutrophil dysfunction is a consequence of induction of the cytoprotective, heme-degrading enzyme heme oxygenase-1 (HO-1) in neutrophil progenitors in bone marrow. In this study, we assessed whether neutrophil dysfunction occurs in humans with malaria and how this relates to hemolysis. We evaluated neutrophil function in 58 Gambian children with \textit{Plasmodium falciparum} malaria [55 (95\%) with uncomplicated disease] and examined associations with erythrocyte count, haptoglobin, hemopexin, plasma heme, expression of receptors for heme uptake, and HO-1 induction. Malaria caused the appearance of a dominant population of neutrophils with reduced oxidative burst activity, which gradually normalized over 8 wk of follow-up. The degree of neutrophil impairment correlated significantly with markers of hemolysis and HO-1 induction. HO-1 expression was increased in blood during acute malaria, but at a cellular level HO-1 expression was modulated by changes in surface expression of the haptoglobin receptor (CD163). These findings demonstrate that neutrophil dysfunction occurs in \textit{P. falciparum} malaria and support the relevance of the mechanistic studies in mice. Furthermore, they suggest the presence of a regulatory pathway to limit HO-1 induction by hemolysis in the context of infection and indicate new targets for therapeutic intervention to abrogate the susceptibility to bacterial infection in the context of hemolysis in humans.


\textit{Plasmodium falciparum} caused an estimated 655,000 deaths and 216 million cases of malaria globally in 2010 (1), but this almost certainly underestimates the indirect health burden (2), which includes increased susceptibility to Gram-negative bacterial infections (3, 4), particularly nontyphoid \textit{Salmonella} (NTS) (3, 5, 6). In areas with high malaria transmission, these indirect effects of malaria infection may explain more than half of the child mortality (2) and community-acquired bacteremia (4). The incidence of NTS closely reflects that of malaria (4, 6, 7), and there is compelling evidence that \textit{P. falciparum} malaria increases susceptibility to NTS bacteremia in humans. In The Gambia, the incidence of NTS bacteremia has declined dramatically over the past 30 y, mirroring the decline in the incidence of malaria (7); this observation has since been confirmed in Kenya (4). In the pre-antibiotic era, malaria therapy for treatment of neurosyphilis was frequently complicated by NTS bacteremia even when NTS infection was otherwise very rare (8), and quinine alone often cured endemic malaria–NTS coinfection (9). NTS bacteremia incidence was found to be more closely related to malaria incidence than to stool carriage of NTS (6), and in Kenyan children, sickle cell trait was found to have a protective effect against bacteremia, which was dependent on the protection it affords against malaria (4). Several studies have shown that susceptibility to NTS is greatest in the context of severe malarial anemia (5, 6), whereas others have found that the greatest risk occurred in children with recent rather than current malaria infection (10, 11).

Most mechanisms that have been proposed to account for the susceptibility to NTS that occurs in malaria involve monocyte and macrophage dysfunction. Malaria may impair monocyte and macrophage function through direct adhesion of infected RBCs (12), through the accumulation of hemoglobin within these cells (13), or by impairment of systemic IL-12 production (14). However, other studies in mice have demonstrated that hemolysis—caused by malaria or in any other way—increases susceptibility to NTS and some other bacterial infections, whereas blood loss alone does not (15–17). We have recently shown in a mouse model of malarial anemia that resistance to \textit{Salmonella typhimurium} is impaired as a result of neutrophil dysfunction (rather than monocyte/macrophage dysfunction) caused by liberation of heme during hemolysis and by induction of the cytoprotective heme-catabolizing enzyme heme oxygenase-1 (HO-1) (18). In this model system, HO-1 induction in myeloid progenitor cells in the bone marrow leads to production of granulocytes with reduced oxidative burst activity, and their mobilization into the blood is enhanced by both hemolysis-derived heme and the response to bacterial coinfection. This results in the accumulation of func-
tionally impaired granulocytes in the circulation that are able to phagocytose *S. typhimurium* but not able to kill the bacteria effectively, providing a new niche for bacterial replication. We found that normal resistance to *S. typhimurium* was restored by inhibition of heme oxygenase (HO) with the competitive inhibitor tin protoporphyrin, a drug that can be used to treat hypobilirubinemia in newborns (19), suggesting that HO inhibitors might represent a novel therapeutic intervention to abrogate the susceptibility to NTS induced by malaria.

Humans and mice with genetic deficiency of subunits of the phagocytic NADPH oxidase, a complex enzyme that catalyzes the generation of superoxide radicals in phagocytic cells, are known to be susceptible to NTS infection (20, 21), and the importance of the neutrophil oxidative burst for killing of serotype-sonopsonized *Salmonella* by blood leukocytes from African children has been demonstrated in vitro (22). Impairment of the neutrophil oxidative burst in humans with malaria would thus be a compelling explanation for susceptibility to NTS bacteremia. In the current study, we investigated whether the same mechanism may apply in humans by examining neutrophil function in a cohort of children with predominantly uncomplicated malaria. Despite the fact that this population would not be considered at high risk of NTS infection, we found that malaria caused a marked abnormality of function in a large proportion of neutrophils, with impairment of oxidative burst capacity but not degranulation. The severity of the impairment of the neutrophil oxidative burst was strongly associated with hemolysis and prior induction of HO-1, but the duration of impairment was much longer than expected, lasting up to 8 wk postinfection.

**Materials and Methods**

**Study subjects and procedures**

The study and all procedures were approved by the Gambian government/ Medical Research Council Laboratories Joint Ethics Committee and the London School of Hygiene and Tropical Medicine Ethics Committee. All human samples were collected with written informed consent from the participant or from the parent or legal guardian of child participants. Between September and December 2010, 58 Gambian children with *P. falciparum* malaria (defined by compatible clinical symptoms and >5000 asexual parasites/μl blood) were recruited within a longitudinal study investigating clinical, immunological, and parasitological factors in mild and severe malaria, details of which have been published (23). Briefly, subjects were recruited, without selection for disease severity, from three peri-urban health centers: the Medical Research Council Gate Clinic, Brikama Health Center, and the Jammeh Foundation for Peace Hospital, Serrekunda. Initial parasitemia (to determine eligibility for inclusion in the study) was estimated from Field’s stained thick blood films and subsequently accurately counted from 50 fields on Giemsa-stained thick blood smears. All children underwent full clinical examination and were managed in accordance with Gambian government guidelines, treated with artemether–lumefantrine for 3 d. Severe malaria was defined using modified World Health Organization criteria (24); severe anemia, defined as hemoglobin concentration ≤ 7 g/dl; severe acidosis defined as blood lactate > 7 mmol/l; cerebral malaria defined as a Blaunty coma score ≤ 2 in the absence of hypoglycemia, with the coma lasting at least for 2 h; severe prostration defined as inability to sit unsupported (children > 6 mo) or inability to suck (children ≤ 6 mo). Children suspected to have concomitant bacterial infections were excluded. For this study, children underwent standardized assessment on the day of presentation (day 0) and on days 7, 28, and 56, and blood was collected for: thick blood film, full blood count (EDTA), immunological assays (sodium hepamin), and RNA (PaxGene tube) (days 0 and 28). On day 0, a thin blood film was prepared, and sickle cell status, blood lactate, and glucose were determined. Blood samples were stored on ice, transported to the laboratory within 2 h of sample collection, and processed within 4 h of collection. Full blood count was performed using a Medonic instrument (Clinical Diagnostics Solutions). Sickle cell status was determined by metabisulfite test and confirmed on cellulose acetate electrophoresis. The proportion of immature neutrophils and hemoglobin (malaria pigment) containing neutrophils and monocytes was determined from the day 0 Giemsa-stained thick blood film. Haptenized whole blood was used for assessment of neutrophil oxidative burst and degranulation (350 μl), intracellular and cell surface flow cytometry (400 μl), and for neutrophil isolation (1.25 ml). On some occasions, there was insufficient blood available to perform all assays. Single blood samples (handled as described earlier) were obtained from six healthy Gambian children and 10 healthy Gambian adults, all without current or recent malaria, recruited from Brefet village where malaria transmission is now extremely low (25).

**Laboratory reagents**

All reagents were obtained from Sigma unless specified otherwise. GEP-expressing *Salmonella enterica* serovar Typhimurium ptv12023 (*S. typhimurium*) was a gift from Prof. David Holden (Imperial College, London, U.K.), grown to late log-phase in Luria–Bertani (LB) broth supplemented with ampicillin, and kept as frozen stock at −70°C in 10% glycerol.

**Neutrophil oxidative burst and degranulation assays**

The neutrophil oxidative burst was assessed in minimally manipulated whole blood using a modification of the assay described by Richardson et al. (26). Briefly, 50-μl aliquots of blood were mixed with 50 μl PMA (final concentration 1 μM) or PBS (as control) and incubated for 15 min at 37°C in a water bath. Next, 25 μl PBS (unstained sample) or staining mixture (anti-CD11b-PE, anti-CD16-PE-Cy7, 7-anti-CD11b (ICRF44; eBioscience), and allylphycocyanin anti-CD15 (VIMC6; Miltenyi Biotec) (unstained and stimulated samples) was added and incubated for 5 min at 37°C in the dark. Ammonium chloride RBC lysis buffer was added for 5 min at room temperature, shielded from light, before washing in PBS and resuspending cells in 1% paraformaldehyde in PBS. Samples were stored at 4°C protected from light and analyzed on the day of collection using a three-laser nine-channel CyAn ADP flow cytometer with Summit 4.3 software (Dako) after calibration of the FL-1 voltage with fluorescent beads (Spherotech). Data were analyzed in FlowJo 7.6 (Tree Star). The magnitude of the oxidative burst was quantified by the rhodamine median fluorescence intensity (MFI), and degranulation was quantified by the fold increase in surface CD11b MFI from the unstimulated to the stimulated sample. Neutrophils were divided into rhodamine+ and rhodamine− populations at the midpoint of the nadir between peaks.

**Flow cytometry for cell surface receptors and intracellular HO-1 expression**

Whole blood was subjected to ammonium chloride RBC lysis and, after washing, cell pellets were resuspended in surface marker Ab mixture (FITC anti-CD91 (A2Mr α-2; AbD Serotec), PE anti-CD16 (CLB-gran11.5; BD Pharmingen), PerCP anti-CD14 (MeP9; BD Pharmingen), allophycocyanin anti-CD163 (215927; R&D Systems)) or a similar mixture instead containing the corresponding manufacturer-matched isotype control Abs for CD91 (mouse IgG1) and CD163 (11711; mouse IgG1). Cells were permeabilized with the Cytofix/Cytoperm (BD Biosciences) before intracellular staining with polyclonal anti–HO-1 (SPA-895; Assay Designs) or an equivalent concentration of polyclonal control rabbit serum (Covance), followed by PE-Cy7–conjugated secondary Ab (F(ab′)2 anti-rabbit IgG; Santa Cruz Biotechnology). The expression of HO-1, CD163, and CD91 were quantified as the ratio of MFI to the respective isotype control Ab for the same sample.

**Neutrophil isolation and *Salmonella* phagocytosis and killing assays**

CD15+ cells were isolated from whole blood, after RBC lysis and labeling with allophycocyanin anti-CD15, using anti-CD15 magnetic beads and MS columns (all from Miltenyi Biotec) according to the manufacturer’s instructions. CD15+ cells were resuspended in RPMI 1640 plus 2 mM L-glutamine at a concentration of 10⁶/ml. GEP-expressing *S. typhimurium* (concentration confirmed by serial dilution) were opsonized in 10% pooled healthy Gambian adult serum (derived from 10 donors, as has been described by others (22)) for 20 min in the dark at room temperature. Neutrophils and *S. typhimurium* were mixed continuously at a ratio of 50:1 at 60 rpm at 37°C. Bacterial counts were assessed at time 0 and 120 min by 10-fold dilutions of aliquots of the neutrophil–*S. typhimurium* suspension diluted directly into PBS 4% paraformaldehyde and analyzing by flow cytometry. To control for autofluorescence and surface binding of bacteria without phagocytosis, control samples were prepared in an identical manner except
that neutrophils and *S. typhimurium* were both fixed with 4% formaldehyde before mixing together. The proportion of cells phagocytosing bacteria was determined by subtraction of the proportion of GFP<sup>+</sup> cells in the fixed-control samples from that in the respective unfixed sample.

**ELISAs**

Plasma levels of *P. falciparum* histidine rich protein-2 (PfHRP-2; Cellabs), hemopexin, haptoglobin (both Genway), C-reactive protein (CRP; R&D Systems), and HO-1 (Enzo Life Sciences) were measured by ELISA. All ELISA assays were performed according to the manufacturer’s instructions, and samples for each assay were performed in a single batch.

**Heme assay**

Total plasma heme (i.e., plasma hemoglobin plus free- and protein bound-heme) was measured using a colorimetric heme assay kit (Quantichrom heme; BioAssay Systems).

**Quantitative RT-PCR**

Total RNA was extracted from PAX tubes using PAXgene blood RNA kits (Qiagen) according to the manufacturer’s instructions and converted into cDNA using a reverse transcription reagent kit (Invitrogen). HMOX1 (141250) gene expression was determined by quantitative RT-PCR (qRT-PCR) on a DNA Engine Opticon (MJ Research) using a TaqMan Probe kit with primers (all Metabion) as described by Hirai et al. (27). 18S rRNA was used as an endogenous reference gene, as its expression has been shown to be stable in acute and convalescent samples from malaria cases regardless of disease severity (23), and was amplified with a commercial kit (RNA primers and VIC labeled probe; Applied Biosystems). Data were analyzed using Opticon Monitor 3 analysis software (Bio-Rad). HMOX1 expression was quantified as the ratio of the transcript number of the HMOX1 to 18S rRNA.

**Estimation of total parasite biomass**

Total parasite biomass was calculated from plasma PfHRP-2 concentration using the method of Dondorp et al. (28). This assumes that PfHRP-2 concentration is an integral of all PfHRP-2 released in preceding rounds of schizogony (when infected erythrocytes rupture to release merozoites) and is therefore a reliable indicator of cumulative hemolysis since the start of the infection (28). We modified the calculation to account for the relatively higher blood volume at lower body weight in small children (29). To account for variation in size of children, parasite biomass was expressed as parasites per kilogram body weight.

**Statistics**

The study was designed to detect a 30% difference in neutrophil oxidative burst activity between samples at day 0 and day 28 with 80% power at the 0.05 significance level, allowing for 15% loss to follow-up. Statistical analysis was performed using PASW Statistics 18 (SPSS). Variables were examined for normality of distribution, and most were found to be nonnormal. Two-tailed nonparametric tests for paired (Wilcoxon matched pairs test) or related repeated measures (Friedman’s two-way ANOVA) were used to compare longitudinal data at different time points, and correlation was tested with Spearman’s rho correlation. To normalize distribution for general linear model analysis, some variables were log<sub>10</sub> transformed or converted to binary variables, as described in the text. Haptoglobin concentrations showed a bimodal distribution and were thus converted to a binary variable (<0.349 mg/ml, the lowest value observed in healthy control samples, or ≥0.349 mg/ml). Sample volumes did not allow for some assays to be performed at time points after day 0, in which case values from six healthy control children were presented for comparison, but not for formal statistical analysis.

**Results**

**Subjects**

Fifty-eight children with *P. falciparum* malaria were recruited to the study, 55 (94.8%) of whom had uncomplicated malaria (Table I). Four children had recurrent episodes of parasitemia during the course of follow-up and were excluded from longitudinal analyses; another 13 children were lost to follow-up or withdrew consent. Thus, at days 7, 28, and 56, the number (%) of children in follow-up were 52 (89.6%), 46 (79.3%), and 41 (70.1%), respectively.

**Prolonged impairment of the neutrophil oxidative burst**

We assessed the PMA-stimulated oxidative burst of neutrophils using a whole-blood flow cytometric assay based on the oxidation of dihydrorhodamine 123 to its fluorescent derivative rhodamine, where the magnitude of the oxidative burst is quantified by the rhodamine fluorescence intensity (26). The assay was modified to allow simultaneous assessment of degranulation based on upregulation of CD11b (32) and surface staining of CD15 to identify neutrophils. We found that neutrophils (Fig. 1A) from subjects with acute malaria (day 0) showed an abnormal, bimodal distribution of oxidative burst activity (Fig. 1B), with distinct populations of rhodamine<sup>hi</sup> and rhodamine<sup>lo</sup> cells, whereas CD11b expression showed a unimodal distribution (Fig. 1C). Overall, neutrophil rhodamine MFI increased significantly over time (Fig. 1D), but in view of the bimodal distribution of neutrophil rhodamine fluorescence, we also compared the proportion of cells that were rhodamine<sup>lo</sup> and the rhodamine MFI of the rhodamine<sup>lo</sup> cells over time. The proportion of rhodamine<sup>lo</sup> cells decreased significantly during the convalescent period (Fig. 1E) but remained above that of healthy controls for at least 56 d; the rhodamine MFI of the rhodamine<sup>lo</sup> cells also significantly increased over time (Fig. 1F). In contrast, there was no evidence of abnormalities in neutrophil degranulation as assessed by CD11b expression (Fig. 1G). Of interest, the rhodamine MFI of the rhodamine<sup>hi</sup> cells was higher on days 0 and 7 after presentation (p = 0.04 and p < 0.001 respectively, Wilcoxon matched pairs test) than on day 56 (Fig. 1H), suggesting that the oxidative burst is primed in these neutrophils (33). These findings are consistent with our observations in mice that hemolysis and infection can prime the oxidative burst of mature, circulating neutrophils while simultaneously mobilizing immature neutrophils with impaired oxidative burst activity from the bone marrow (18). However, the duration of these neutrophil abnormalities after *P. falciparum* infection was longer than we expected. We assessed whether a similar abnormality was present in monocytes in peripheral blood (Supplemental Fig. 1A) but found that the oxidative burst appeared to be slightly enhanced on day 0 compared with other time points (Supplemental Fig. 1B, 1C).

**Hemolysis and neutrophil dysfunction**

We have previously shown that hemolysis-derived heme impairs neutrophil function during malaria infection in mice through two related mechanisms: mobilization of functionally immature neutrophils from bone marrow, and impairment of the oxidative burst capacity of developing neutrophils due to HO-1 induction in bone marrow progenitors (18). RBC destruction in malaria is multifactorial, but the severity of intravascular hemolysis can be inferred from levels of the plasma proteins haptoglobin and hemopexin (34), which provide sequential lines of defense against heme-mediated toxicity by binding cell-free hemoglobin and cell-free heme, respectively (35). Only once haptoglobin is depleted do levels of hemopexin begin to fall, indicating that heme is being released from cell-free hemoglobin (36).

To assess the extent of hemolysis in study participants, we measured erythrocyte count and total parasite biomass (Table I), total plasma heme (Fig. 2A), haptoglobin (Fig. 2B), and hemopexin (Fig. 2C) and examined their correlation with the proportion and function of rhodamine<sup>lo</sup> neutrophils (Table II). Although only 13 (22.4%) children were actually anemic by Gambian reference standards (Hb <9.5 g/dl) (30), total plasma heme levels were significantly greater on day 0 than on day 28 (Fig. 2A). Levels of haptoglobin showed a bimodal distribution (Fig. 2B), consistent with the expected depletion of haptoglobin by hemolysis in some
Table I. Demographic, clinical, and laboratory characteristics at recruitment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>n (%)</th>
<th>Median (IQR)</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>36 (62)</td>
<td>11.5 (9.98–12.5)</td>
<td>9.5–14.4³</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Female</td>
<td>22 (38)</td>
<td>4.26 (3.83–4.66)</td>
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</tr>
<tr>
<td></td>
<td>Mandingo</td>
<td>25 (43)</td>
<td>76.9 (73.5–80.7)</td>
<td>67.8–90.0²</td>
</tr>
<tr>
<td></td>
<td>Fula</td>
<td>10 (17)</td>
<td>8.50 (6.74–10.3)</td>
<td>4.1–11.1³</td>
</tr>
<tr>
<td></td>
<td>Wolof</td>
<td>7 (12)</td>
<td>74.8 (66.3–83.0)</td>
<td>35–75²</td>
</tr>
<tr>
<td></td>
<td>Manjago</td>
<td>5 (9)</td>
<td>1.39 (0.57–2.75)</td>
<td>0–10⁶</td>
</tr>
<tr>
<td></td>
<td>Jola</td>
<td>5 (9)</td>
<td>5.32 (2.57–10.8)</td>
<td>0–10⁶</td>
</tr>
<tr>
<td>Age (y)</td>
<td>Uncomplicated</td>
<td>55 (95)</td>
<td>20.0 (5.0–50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>3 (5)</td>
<td>92,800 (28,200–219,000)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prostration</td>
<td>2</td>
<td>1.23 × 10⁻¹⁰⁵ (5.21 × 10⁻¹⁰⁵ to 2.14 × 10⁻¹⁰⁵)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactic acidosis</td>
<td>1</td>
<td>106.4 (64.5–234.3)</td>
<td></td>
</tr>
<tr>
<td>Sickle cell screen</td>
<td>Negative</td>
<td>53 (91)</td>
<td>2.0 (1.6–2.45)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>1 (2)</td>
<td>2.0 (1.6–2.45)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Not done</td>
<td>4 (7)</td>
<td>2.0 (1.6–2.45)</td>
<td></td>
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<tr>
<td>Hemoglobin, g/dl</td>
<td></td>
<td>58</td>
<td>4.26 (3.83–4.66)</td>
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</tr>
<tr>
<td>Erythrocyte count, ×10¹²/l</td>
<td></td>
<td>58</td>
<td>76.9 (73.5–80.7)</td>
<td>67.8–90.0²</td>
</tr>
<tr>
<td>Mean corpuscular volume, fl</td>
<td></td>
<td>56</td>
<td>8.50 (6.74–10.3)</td>
<td>4.1–11.1³</td>
</tr>
<tr>
<td>Leukocyte count, ×10⁷/l</td>
<td></td>
<td>57</td>
<td>1.39 (0.57–2.75)</td>
<td>0–10⁶</td>
</tr>
<tr>
<td>Percent neutrophils</td>
<td></td>
<td>58</td>
<td>5.32 (2.57–10.8)</td>
<td>0–10⁶</td>
</tr>
<tr>
<td>Percent bandsd</td>
<td></td>
<td>58</td>
<td>1.22 (0.42–4.08)</td>
<td></td>
</tr>
<tr>
<td>Percent immature³,⁴,⁵</td>
<td></td>
<td>58</td>
<td>20.0 (5.0–50)</td>
<td></td>
</tr>
<tr>
<td>Percent pigmented²</td>
<td></td>
<td>58</td>
<td>92,800 (28,200–219,000)</td>
<td></td>
</tr>
<tr>
<td>Percent of monocytes with pigment</td>
<td></td>
<td>58</td>
<td>1.23 × 10⁻¹⁰⁵ (5.21 × 10⁻¹⁰⁵ to 2.14 × 10⁻¹⁰⁵)</td>
<td></td>
</tr>
<tr>
<td>Parasite density, parasites/μl</td>
<td></td>
<td>57</td>
<td>106.4 (64.5–234.3)</td>
<td></td>
</tr>
<tr>
<td>Parasite biomass, parasites/kg</td>
<td></td>
<td>55</td>
<td>2.0 (1.6–2.45)</td>
<td></td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td></td>
<td>56</td>
<td>2.0 (1.6–2.45)</td>
<td></td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td></td>
<td>42</td>
<td>2.0 (1.6–2.45)</td>
<td></td>
</tr>
</tbody>
</table>

*Data were not available for every variable for every subject.

³90% reference interval for Gambian children aged 6–12 y (30), where available, for laboratory parameters.

²General pediatric reference interval (31), where available, for laboratory parameters.

³Percentage of all neutrophils.

⁴Neutrophil precursors less mature than band forms.

ASS, Hemoglobin AS heterozygote; IQR, interquartile range.

subjects (36), but also increased production of haptoglobin as part of the acute-phase response in other subjects (37, 38). Hemopexin levels, however, were relatively normally distributed and very similar to healthy controls (Fig. 2C), suggesting that in these subjects with predominantly uncomplicated malaria, hemolysis does not liberate sufficient cell-free heme to deplete plasma hemopexin (35, 36).

Because rhodamine<sub>Lo</sub> cells may be similar to the functionally immature granulocytes released into the circulation during malaria and NTS infection in mice (18), we assessed whether their frequency in any of the study subjects, our analysis of the HO-1 pathway was restricted to parameters measurable in peripheral blood, namely plasma HO-1, whole-blood HMOX1 gene expression, and HO-1 protein expression in peripheral blood cells. Also, because the induction of cellular HO-1 by haptoglobin–hemoglobin or heme–hemopexin complexes depends on the presence of surface receptors for their uptake (CD163 and CD91, respectively) (37, 40, 41), we examined CD163 and CD91 expression on monocytes and neutrophils.

Factors associated with HO-1 induction

In malaria-infected mice, we found that heme-mediated HO-1 induction in neutrophil progenitors in bone marrow was necessary to impair the oxidative burst of developing neutrophils (18). Because there were no clinical indications for bone marrow aspiration in any of the study subjects, our analysis of the HO-1 pathway was restricted to parameters measurable in peripheral blood, namely plasma HO-1, whole-blood HMOX1 gene expression, and HO-1 protein expression in peripheral blood cells. Also, because the induction of cellular HO-1 by haptoglobin–hemoglobin or heme–hemopexin complexes depends on the presence of surface receptors for their uptake (CD163 and CD91, respectively) (37, 40, 41), we examined CD163 and CD91 expression on monocytes and neutrophils.
As previously reported in mice (18) and humans (42), HO-1 expression (assessed by fluorescence intensity) in circulating neutrophils was not increased in acute malaria infection compared with convalescence (data not shown). In contrast, monocyte HO-1 expression was higher on day 0 than on days 7 or 28 (Fig. 3A). Plasma HO-1 was higher in subjects on day 0 than in healthy control children (Fig. 3B), and as we have previously reported (42), whole-blood HMOX1 expression was significantly higher on day 0 than after recovery on day 28 (Fig. 3C).

In control subjects, CD163 and CD91 were expressed on the surface of monocytes but were not detectable on neutrophils (Fig. 3D). In children with malaria, monocyte CD163 expression was significantly lower at day 0 than on days 7 and 28 (Fig. 3E), whereas CD163 remained undetectable on neutrophils from most subjects (Fig. 3F). CD91 expression did not change significantly over time on monocytes (Fig. 3G) or neutrophils (Fig. 3H), although when all subjects were considered together, there did appear to be very low level CD91 expression on neutrophils at all time points.

To explore further the likely pathways of HO-1 induction during malaria infection, we constructed a simple conceptual model (Fig. 4), beginning with the malaria parasite as the cause of hemolysis, inflammation, and tissue hypoxia/ischemia (43)—all of which may induce HO-1 expression (44)—and analyzed univariate correlations between the various measures of HO-1 induction. As expected, parasite biomass was strongly correlated with total plasma heme and CRP. Plasma HO-1 correlated much more strongly with CRP and lactate than with plasma heme, supporting the idea that it...
Levels in six healthy control children are also shown. Distribution of plasma hemopexin levels at day 0, comparison, levels in six healthy control children are also shown. 

**Neutrophil oxidative burst and prior HO-1 induction**

In mice, we had observed that suppression of the oxidative burst of circulating neutrophils by hemolysis required the release of immature neutrophils from bone marrow, requiring either a lag time or an additional stimulus (such as NTS infection) to cause these cells rapidly to enter the circulation (18). Having observed that an abnormal population of neutrophils was present for a prolonged period of time after *P. falciparum*, we looked for evidence of an association between HO-1 induction on day 0 and neutrophil oxidative burst on day 7. We used the ratio of day 0 to day 28 whole-blood *HMOX1*(*HMOX1*day0/day28) expression as an indicator of induction in acute malaria. We found a significant negative correlation between *HMOX1*day0/day28 and the rhodamine MFI of rhodamineLo neutrophils on day 7 (Spearman’s correlation coefficient = −0.352, *p* = 0.028, *n* = 39). There was no significant correlation with the proportion of rhodamineLo cells on day 7 (Spearman’s correlation coefficient = −0.101, *p* = 0.542, *n* = 39).

Although the kinetics of the process of HO-1 induction, suppression of oxidative burst capacity in developing neutrophils, and subsequent release of the functionally immature neutrophils into the circulation are unknown, the observed association between HO-1 induction during acute disease and neutrophil dysfunction during early convalescence is consistent with this sequence of events.

**Salmonella phagocytosis and killing**

As we have observed that neutrophil killing (but not phagocytosis) of *S. typhimurium* is defective in malaria-infected mice (18), we assessed the ex vivo killing and phagocytosis of serum-opsonized *S. typhimurium* by neutrophils isolated from whole blood of subjects and controls from whom sufficient blood remained after subjects and controls from whom sufficient blood remained after

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**Table II. Association of neutrophil dysfunction with hemolysis on day 0**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Percent RhodamineLo Neutrophils</th>
<th>Rhodamine MFI of RhodamineLo Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n*</td>
<td>Correlation Coefficient</td>
</tr>
<tr>
<td>Parasite biomass/kg</td>
<td>53</td>
<td>0.208</td>
</tr>
<tr>
<td>Erythrocyte count</td>
<td>56</td>
<td>−0.001</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>55</td>
<td>0.004</td>
</tr>
<tr>
<td>Total plasma heme</td>
<td>54</td>
<td>0.222</td>
</tr>
<tr>
<td>CRP</td>
<td>44</td>
<td>0.350</td>
</tr>
<tr>
<td>Immature neutrophil percent</td>
<td>56</td>
<td>0.232</td>
</tr>
<tr>
<td>Pigmented neutrophil percent</td>
<td>56</td>
<td>−0.017</td>
</tr>
</tbody>
</table>

*Indicators of hemolysis, inflammation, and neutrophil characteristics were assessed for correlation with the proportion of rhodamineLo neutrophils and with the rhodamine MFI of rhodamineLo neutrophils on day 0 using Spearman’s correlation.

*Data were not available for every variable for every subject.
heme, or parasite biomass (data not shown). Bacterial phagocytosis, determined by flow cytometric analysis of the proportion of GFP+ neutrophils after 15-min coculture (Fig. 5B), did not vary significantly over time after infection (Fig. 5C). However, phagocytosis at day 0 was inversely correlated with parasite biomass ($n = 26$, Spearman’s correlation coefficient $= -0.512$, p = 0.008), and with total plasma heme ($n = 28$, Spearman’s correlation coefficient $= -0.441$, p = 0.019) (Fig. 5D). Taken together with the neutrophil oxidative burst assay data (Fig. 1F, 1H), these data indicate that although there may be some degree of (heme-mediated) priming of neutrophil function in children with acute malaria (day 0), which enhances bacterial killing during acute illness (47), phagocytosis of S. typhimurium by circulating neutrophils becomes increasingly impaired with increasing parasite burden and increasingly severe hemolysis, and the ability of neutrophils to kill S. typhimurium once they are phagocytosed might also become impaired.

Discussion
Although the association between malaria infection and susceptibility to NTS bacteremia has been recognized for almost a century (9), the mechanism has been elusive. The strongest association is with severe malarial anemia (5, 6), and at least two other conditions associated with hemolytic anemia—sickle cell disease (48) and acute bartonellosis (49)—also predispose to NTS bacteremia. This is likely a result of both the nature of the defect in host defense induced by malaria and the prevailing epidemiology of invasive bacterial infection: NTS is one of the most common causes of bacteremia in Sub-Saharan Africa (50, 51). In a mouse model, we recently showed that hemolysis due to malaria or phenylhydrazine treatment impaired resistance to S. typhimurium, which could be recapitulated by treatment with hemin and abrogated by treatment with the HO inhibitor tin protoporphyrin (18). We found that bacteria accumulated in circulating neutrophils and that these neutrophils were defective in killing S. typhimurium, associated with impairment of their oxidative burst response, which is an essential mechanism for killing S. typhimurium (21). This was due to heme-mediated induction of HO-1 in granulocyte precursors in bone marrow, causing neutrophils leaving the bone marrow to have a reduced capacity to mount an effective oxidative burst. It is not known whether bacteria accumulate preferentially in neutrophils in humans with malaria and NTS coinfections, but in the pre-antibiotic era, neutrophils and NTS were often found colocalized in abscesses that formed at the site of i.m. quinine injection in coinfected individuals (9). Although neutrophil function has not been extensively studied in malaria, there are several case reports...
of patients with severe malarial hemolysis spontaneously developing fungal sepsis (52–54), which is typically associated with neutropenia and neutrophil dysfunction.

The current study was designed to determine whether Gambian children with *P. falciparum* malaria have evidence of neutrophil dysfunction similar to that observed in mice infected with *Plasmodium yoelii* 17XNL. We hypothesized that the neutrophil oxidative burst would be impaired in children with malaria, and the severity of this impairment would be related to hemolysis and HO-1 induction. However, we also predicted that the impairment of neutrophil function would be relatively mild because declining malaria transmission in The Gambia has led to a decrease in the incidence of severe malarial anemia and malaria–NTS coinfection (7). Overall, our results are consistent with our hypothesis: the oxidative burst activity of circulating neutrophils was profoundly abnormal in subjects with acute *P. falciparum* malaria and most severely impaired in children with the highest parasite burdens and greatest hemolysis, albeit the magnitude of this impairment did not translate into a clinically significant defect in neutrophil killing of *S. typhimurium* in vitro. We also found that these abnormalities persisted for at least 56 d and that bacterial phagocytosis and killing appeared to deteriorate during the early convalescent period (up to 28 d), findings that may be consistent with descriptions of increased susceptibility to NTS bacteremia in children who have recently had malaria (6, 10) and the gradual emergence of dysfunctional neutrophils from bone marrow after HO-1 induction during the acute infection (18). To facilitate comparison with our studies in mice (18), we assessed neutrophil function using PMA as the stimulus for the oxidative burst. Although this is not a physiological stimulus, the advantages of this method are i) that it produces a strong oxidative burst (55, 56), which is clearly distinguished from any low-level activation caused by malaria infection per se; ii) it is not dependent on phagocytosis (which might also be impaired by malaria) (55, 56); and iii) variations in the magnitude of the PMA-induced oxidative burst are directly related to the ability of humans to survive infections (57).

Consistent with data from mice and humans (18, 42, 58), we observed induction of HO-1 during acute malaria. Although dissecting the causal and consequential pathways of HO-1 induction is difficult in an observational study, we constructed a conceptual model of likely pathways leading to HO-1 induction based on existing literature (Fig. 4) (44) and used this model to guide our statistical analysis. Plasma HO-1 levels correlated more strongly with plasma lactate and CRP concentrations than with plasma heme concentrations, suggesting that plasma HO-1 may be predominantly a response to inflammation and hypoxia and a marker of cell damage. As noted previously, intracellular HO-1 protein expression was not significantly upregulated in acute malaria in circulating neutrophils (42), presumably because they lack CD163 expression, whereas monocytes did show evidence of increased HO-1 protein expression in acute malaria. However, there was not a significant univariate association between total plasma heme concentration and HO-1 expression in monocytes, which could be explained statistically by the reduced levels of monocyte surface CD163 expression in acute malaria. This explanation is fully consistent with the subjects in this study having only mild hemolysis [most had hemoglobin values within the normal range for their age (30), none had severe malarial anemia, and only half had low haptoglobin levels] and with the assumption (as hemopexin levels were not depleted) that very little of the total circulating heme represents cell-free heme. In this case, HO-1 induction due to hemolysis would be expected to proceed predominantly through CD163-mediated uptake of haptoglobin–hemoglobin complexes, and reduction in surface CD163 would be expected to limit HO-1 induction (45). In contrast, severe hemolysis would be expected to generate cell-free heme and lead to HO-1 induction and heme catabolism in cells expressing the surface receptor (CD91) for heme–hemopexin complexes, which appears to be invariantly expressed during infection. Indeed, we previously found elevated carboxyhemoglobin levels, an indirect measure of HO activity, only in children with severe malarial anemia suggesting that heme catabolism is constrained in acute malaria and only detectably increased in cases with the most severe hemolysis (59). It is conceivable that by reducing CD163 expression in the context of infection, monocytes are rendered relatively resistant to HO-1 induction by hemolysis, perhaps preventing HO-1–mediated impairment of their normal inflammatory responses (45, 60). However, it is currently unknown whether either CD163 or CD91 expression is required for HO-1 induction in immature myeloid cells and their progenitors in human bone marrow. If HO-1 induction in bone marrow is responsible for the observed neutrophil dysfunction [as it is in mice (18)], either it may be independent of CD163 or CD163 may not be downregulated in the bone marrow to the same extent as in blood monocytes. We did not examine the effect of *HMOX1* promoter (GT)n length polymorphisms in this study because the majority of subjects had uncomplicated malaria with mild hemolysis, and we expected that under these circum-
phagocytosis expressed as the percentage of GFP+ neutrophils after 15-min incubation. The percentage of GFP+ cells was calculated by subtracting the proportion of GFP+CD15+ cells in formaldehyde fixed samples from that in unfixed samples. Statistical comparison using Friedman’s two-way ANOVA for all subjects with valid data at all time points, \( n = 18 \). Data from control subjects shown for comparison. 

\( P = 0.015 \)

\( \text{Bacterial killing (\%)} \)

\( \text{Days after presentation} \)

\( \text{C} \) Phagocytosis of \( S. \text{typhimurium} \) by neutrophils isolated on days 0, 7, 28, and 56 after presentation with \( P. \text{falciparum} \) malaria. The percentage of GFP+CD15+ cells was determined in samples where both neutrophil population, the oxidative burst of the rhodamineHi neutrophil population was higher on days 0 and 7 than at later time points, and enhanced bactericidal activity among rhodamineHi cells may initially compensate for the lack of killing among the rhodamineLo population, particularly if the rhodamineHi cells showed preferential phagocytosis of the opsonized \( S. \text{typhimurium} \). Unfortunately, our assays could not simultaneously assess phagocytosis and oxidative burst in the same cells. We predict, however, that bacterial killing would be seriously impaired in children with severe hemolytic anemia.

The acquired defect of neutrophil function that we observed in children with malaria in this study might be considered analogous to the neutrophil defect observed in female carriers of X-linked chronic granulomatous disease, where around 50% of neutrophils have defective oxidative burst activity due to random inactivation of the X-chromosome but there is not increased susceptibility to infection (61), and in vitro bactericidal activity may be normal (62). However, in some carriers, inactivation of the X-chromosome becomes skewed, and when <15% of neutrophils are able to make a normal oxidative burst, susceptibility to infections is markedly increased (20). Consistent with this, in a large European registry of chronic granulomatous disease patients, \( S. \text{mononella} \) has been reported as by far the most common cause of septicemia (whereas fungi and \( S. \text{aureus} \) are the most common causes of chronic lung and deep tissue infections, due to the persistent defect in oxidative burst activity) (20). We propose that in patients with malaria, a threshold proportion of abnormal neutrophils in blood may be required to produce susceptibility to NTS, and when this threshold is exceeded, the degree of susceptibility may then also be determined by the magnitude of the impairment of oxidative burst capacity. Factors that may determine whether the proportion of dysfunctional neutrophils exceeds this putative threshold may include the duration of infection and the severity of the inflammatory response, which may both influence the mobilization of dysfunctional neutrophils from bone marrow (18); the severity of hemolysis, as cell-free heme itself promotes neutrophil mobilization (18); and possibly host factors such as age and genetic background. The strong correlation we observed between impaired neutrophil oxidative burst and total parasite biomass allows us to predict that children with high parasite burden (who are also most likely to have severe hemolysis) would have the most impaired oxidative burst. These children may also have depleted hemopexin levels and accumulate cell-free heme, which can itself mobilize neutrophils from bone marrow (18), perhaps increasing the proportion of abnormal

\( D \) Correlation of phagocytosis (on day 0) with parasite biomass on day 0 (left-hand panel, \( n = 26 \)) and total plasma heme on day 0 (right-hand panel, \( n = 28 \)).
neutrophils above a threshold required to induce susceptibility to NTS.

The prolonged duration of abnormal neutrophil oxidative burst activity, extending up to 8 wk after acute infection in some subjects, was unexpected. This is unlikely to be due to antimalarial treatment, because artemisinin-based treatments cause mild enhancement of the oxidative burst and suppression of phagocytosis, the opposite of what we observed (63). Two possible explanations are the effect of persisting hemozoin and fundamental alteration in myelopoiesis. Hemozoin is an insoluble heme polymer, the end product of hemoglobin digestion inside the parasitized red cell, and is able to induce HO-1 (and impair the oxidative burst in phagocytes) but is not catabolized (13, 64). Prolonged persistence of hemozoin in phagocytic cells in the bone marrow (65) could cause prolonged HO-1 induction and abnormal development of neutrophils (18). Associated with this or through separate mechanisms, a novel myeloid progenitor phenotype may be generated in P. falciparum malaria, as has been observed in rodent malaria infection (66), and may lead to sustained abnormal granulopoiesis.

The major limitations of our study are that most subjects had relatively mild hemolysis and that we did not have bone marrow samples to confirm HO-1 induction in neutrophil progenitors. To recruit a significant number of subjects with severe malarial hemolysis would require a much larger study, probably conducted in a higher-transmission setting. To study prospectively whether the severity of neutrophil dysfunction at recruitment correlated with susceptibility to NTS bacteremia during convalescence would require an even larger and more logistically complex study. To obtain bone marrow aspirates from children with malaria would be difficult to justify ethically unless appropriate sedation and analgesia could be provided without additional risk of complications.

Nevertheless, our findings have a number of important implications. First, we show that the oxidative burst capacity of a large proportion of neutrophils is markedly abnormal in children with P. falciparum malaria, supporting the translation of findings in a mouse model (18). Second, neutrophil function recovers only very slowly over the 2 mo after treatment, providing an explanation for the association of susceptibility to NTS with recent malaria (10, 11). In the mouse model, hemolysis-induced neutrophil dysfunction could be abrogated by competitive inhibition of HO with tin protoporphyrin (18), but using this treatment in acute malaria would be challenging because HO-1 is also important for tolerance to cytotoxic effects of cell-free heme in mouse models (58, 67). Alternative therapeutic strategies would be administration of tin protoporphyrin upon completion of antimalarial treatment, with the aim of restoring neutrophil function during convalescence and preventing the susceptibility to NTS caused by recent malaria, or prioritization of children at greatest risk of persistent neutrophil dysfunction for prophylactic antibiotic treatment. Third, we propose the downregulation of the haptoglobin receptor CD163 on the surface of blood monocytes during acute P. falciparum malaria as a novel host-protective homeostatic response to hemolysis and inflammation, which may prevent HO-1 induction from impairing monocyte function. Further experimental studies are needed to confirm the effects of manipulating CD163 expression during infections, but manipulation of this axis would hold promise for both the modulation of inflammation and optimization of iron reutilization during chronic infections.

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Disclosures

The authors have no financial conflicts of interest.

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SUPPLEMENTARY FIGURE 1. *P. falciparum* malaria enhances monocyte oxidative burst. (A) Representative FACS plots showing the gating of the monocyte population based on forward scatter and side scatter characteristics followed by selection of single cells based on pulse width and forward scatter. (B) Rhodamine fluorescence of unstimulated (filled histogram) and PMA-stimulated monocytes (unfilled histogram) on days 0, 7, 28 and 56 after presentation with *P. falciparum* malaria. Representative plots from a healthy control child are also shown for comparison. (C) Longitudinal analysis of monocyte oxidative burst compared using Friedman’s two way ANOVA for all subjects with valid data at all time points, n=33. Healthy controls are also shown for comparison, but not included in the statistical analysis. Horizontal lines represent medians.