Published in final edited form as:

Am J Hematol. 2011 February ; 86(2): 155–162. doi:10.1002/ajh.21933.

Mechanisms of erythropoiesis inhibition by malarial pigment and malaria-induced proinflammatory mediators in an in vitro model

Gordon A. Awandare^{1,2}, Prakasha Kempaiah³, Daniel O. Ochiel¹, Paolo Piazza¹, Christopher C. Keller^{1,4}, and Douglas J. Perkins^{3,*}

¹Department of Infectious Diseases and Microbiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania

²Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon-Accra, Ghana

³Department of Internal Medicine, Center for Global Health, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

⁴Laboratory of Human Pathogens, Lake Erie College of Osteopathic Medicine, Erie, Pennsylvania

Abstract

One of the commonest complications of *Plasmodium falciparum* malaria is the development of severe malarial anemia (SMA), which is, at least in part, due to malaria-induced suppression of erythropoiesis. Factors associated with suppression of erythropoiesis and development of SMA include accumulation of malarial pigment (hemozoin, PfHz) in bone marrow and altered production of inflammatory mediators, such as tumor necrosis factor (TNF)- α , and nitric oxide (NO). However, studies investigating the specific mechanisms responsible for inhibition of red blood cell development have been hampered by difficulties in obtaining bone marrow aspirates from infants and young children, and the lack of reliable models for examining erythroid development. As such, an in vitro model of erythropoiesis was developed using CD34+ stem cells derived from peripheral blood to examine the effects of PfHz, PfHz-stimulated peripheral blood mononuclear cell (PBMC)-conditioned media (CM-PfHz), TNF- α , and NO on erythroid cell development. PfHz only slightly suppressed erythroid cell proliferation and maturation marked by decreased expression of glycophorin A (GPA). On the other hand, CM-PfHz, TNF- α , and NO significantly inhibited erythroid cell proliferation. Furthermore, decreased proliferation in cells treated with CM-PfHz and NO was accompanied by increased apoptosis of erythropoietin-stimulated CD34+ cells. In addition, NO significantly inhibited erythroid cell maturation, whereas TNF- α did not appear to be detrimental to maturation. Collectively, our results demonstrate that PfHz suppresses erythropoiesis by acting both directly on erythroid cells, and indirectly via inflammatory mediators produced from PfHz-stimulated PBMC, including TNF- α and NO.

*Correspondence to: Douglas J. Perkins, Department of Internal Medicine, Center for Global Health, University of New Mexico Health Sciences Center, MSC10-5550, Albuquerque, NM 87131-0001. dperkins@salud.unm.edu or gawandare@hotmail.com. Daniel O. Ochiel is currently at Harvard School of Public Health, Boston, MA

Introduction

Severe malarial anemia (SMA) is the most common clinical manifestation of severe *Plasmodium falciparum* malaria in infants and young children, and accounts for the greatest amount of global malaria-related morbidity and mortality [1]. Although SMA can result from hemolysis of parasitized and non-parasitized red blood cells (RBCs), our recent investigations demonstrate that pediatric SMA in holoendemic regions results largely from suppression of erythropoiesis [2,3]. In addition, deficient erythropoietin (Epo) production does not appear to be the major cause of decreased erythropoiesis, since appropriately increased levels of Epo have been reported in children with malarial anemia [4–7]. Moreover, suppressed erythropoietic responses in children with *P. falciparum* are rapidly reversible following parasite clearance [4,6], suggesting that malaria-induced mediators may be responsible for this phenomenon.

Our recent studies, as well those of others, demonstrate that SMA in children residing in holoendemic *P. falciparum* transmission areas is associated with high circulating concentrations of hemozoin (PfHz), as well as elevated levels of PfHz-containing monocytes [8,9]. Furthermore, these studies show a direct correlation between PfHz levels and suppression of erythropoiesis [8,9], suggesting that this may be one important mechanism by which malarial parasite products contribute to the pathogenesis of SMA. Since PfHz is found both free in circulation and deposited intracellularly in the bone marrows of children with SMA [10,11], the mechanism(s) responsible for decreased erythropoietic responses could be either direct, or indirect through the release of soluble mediators from PfHz-containing cells. Phagocytosis of PfHz promotes over-production of pro-inflammatory mediators, such as tumor necrosis factor (TNF)- α and nitric oxide (NO) that may be involved in the suppression of erythropoiesis [8,12–18]. Although children with malarial anemia have elevated levels of TNF- α and NO and earlier studies have demonstrated that both of these mediators are potent inhibitors of erythropoiesis, [14,17,19–26], the mechanism(s) through which these soluble mediators exert their action are largely undetermined.

Investigation of the molecular mechanisms involved in suppression of the erythropoietic response in children with malarial anemia have been hampered by difficulties in obtaining sufficient quantities of erythroid progenitors from the bone marrow of severely anemic children, and the lack of a simple, reliable and reproducible in vitro model. Previous studies demonstrated that CD34+ hematopoietic stem cells can be cultivated in liquid media and induced to differentiate into RBCs [27,28]. Based on these principles, we developed an in vitro model of erythropoiesis in which CD34+ hematopoietic stem cells were isolated from large quantities of peripheral blood obtained from healthy, U.S. donors. Using this in vitro model and a combination of molecular, biochemical, and flow cytometric methods, we evaluated the effects of PfHz, synthetic Hz (sHz), supernatants from PfHz-stimulated peripheral blood mononuclear cells (PBMCs), TNF- α , and NO on the proliferation, survival, and differentiation of CD34+ stem cells into mature erythroid cells. Results from these studies demonstrate that both PfHz and PfHz-induced inflammatory mediators play important roles in suppressing erythropoiesis.

Materials and Methods

Isolation of CD34+ cells

PBMCs were isolated using Ficoll-hypaque from donor leukopack samples (50 mL) obtained from the University of Pittsburgh Medical Center blood bank under protocols approved by the Ethics Committee of the University of Pittsburgh Institutional Review Board. PBMCs were washed twice and re-suspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.6% anticoagulant citrate dextrose formula A (ACD-A). Hematopoietic progenitor cells (CD34+) were enriched by two cycles of positive selection using anti-CD34 microbe-ads and magnetic cell sorting on Midi-MACS columns (Direct CD34 progenitor cell isolation kit, Miltenyi Biotec, Auburn, CA). The number of viable CD34+ cells obtained was determined by the trypan blue exclusion method.

Preparation of hemozoin

P. falciparum (PfD6, country of origin Sierra Leone, West Africa; obtained from America type culture center, ATCC) was cultivated in the laboratory with human type O+ RBCs according to principles of Trager and Jensen [29], and harvested when the majority of parasites were at the PfHz-rich late asexual stages (i.e., late trophozoites and early schizonts). Parasitized RBCs were then lysed and PfHz was isolated, dried and resuspended at 1.0 mg/mL as described previously [30]. Synthetic Hz, (β -hemin, sHz) was prepared from hemin chloride (Sigma, St Louis, MO) and also resuspended at 1.0 mg/mL per our previous methods [30]. Endotoxin levels in all PfHz and sHz preparations were determined to be <0.125 EU/mL (i.e., <0.025 ng/mL; Limulus amoebocyte lysate test, BioWhittaker, Walkersville, MD), indicating that the preparations were free of endotoxins contamination.

Erythroid cell growth media

Culture media used in this study were based on previously described methods [27,28] that have been modified for selectively optimizing erythroid development with negligible contamination from other cell lineages. Basic culture medium was composed of Iscove's Modified Dulbecco's Medium (IMDM, Gibco-Invitrogen, Carlsbad, CA) containing 15% BIT (mixture of 5% BSA, 50 μ g/mL bovine pancreatic insulin and 1.0 mg/mL human transferrin; Stem cell technology, Canada), 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO). Primary culture medium was prepared from the basic media by adding a combination of cytokines: 10 ng/mL recombinant human (rh) interleukin (IL)-3, 10 ng/mL rh IL-6, and 100 ng/mL rh stem cell factor (SCF) (R&D systems, Minneapolis, MN). To induce erythropoiesis, Rh Epo (1.0 U/mL, R&D systems, Minneapolis, MN) was added to the primary culture media to obtain the secondary culture media.

Primary cell culture

Isolated CD34+ cells were plated in a 24-well plate at 1×10^5 cells/well in primary culture media and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 5% O₂ for 3 days.

At the end of the incubation period, cells were harvested, washed twice in IMDM and the number of viable cells were determined by the trypan blue exclusion method.

Secondary cell culture and treatment with PfHz, sHz, TNF- α , and NO

Cells obtained from the primary culture were re-plated at 15×10^3 cells/well in secondary media alone (Epo Control), or in combination with PfHz (10 μ g/mL), sHz (10 μ g/mL), rh TNF- α (10 ng/mL and 100 ng/mL), and nitric oxide donors, DETANONOate (50 μ M and 100 μ M) and PAPANONOate (50 μ M and 100 μ M; Cayman Chemical, Ann Arbor, MI), respectively. Additional secondary cultures were established using basic media that had been pre-conditioned for 24 hr with unstimulated PBMC (CM-Con), PfHz-stimulated PBMC (CM-PfHz) or sHz-stimulated PBMC (CM-sHz), i.e., 1×10^6 PBMC/mL with 10 μ g/mL of PfHz or sHz. Additional cells were cultured in primary media alone (No Epo) to serve as a negative control for erythropoiesis. Cultures were maintained for 11 additional days to allow appropriate erythropoiesis. In all experiments, the CD34+ cells were exposed to the inflammatory mediators or PBMC-conditioned media for 3 days, after which half of the culture media were replaced with freshly prepared media every 2 days to maintain optimal nutrient balance.

Cell proliferation assay

Cell proliferation was assessed using a methylthiazoletetrazolium (MTT)-based assay. The assay is based on the ability of viable cells to metabolize MTT, forming an insoluble formazan dye which is then quantified by spectrophotometry. Briefly, on the indicated days, cells were resuspended by gentle titration and 200 μ L of culture from each well was transferred to a corresponding well in a 96-well plate. Twenty microliters of MTT (dissolved at 5 mg/mL in PBS) was added and incubated under culture conditions for 4 hrs. The synthesized formazan was solubilized using dimethyl sulfoxide (DMSO) and its absorbance was read at 570 nm. Relative cell proliferations across different conditions were determined by expressing absorbances as percent of Epo Con.

Apoptosis assay

Cellular apoptosis was assessed by quantifying the concentrations of nucleosomes in cell lysates (early-stage apoptosis) and culture supernatants (late-stage apoptosis) using a cell death detection ELISA (Roche Diagnostics, Germany) according to the manufacturer's recommendations. Briefly, cells were re-suspended by gentle titration and 100 μ L of culture was transferred to a 96-well plate. The plate was centrifuged at 1,000 rpm for 10 min, and the supernatants harvested for nucleosome ELISA. The cell pellet was gently washed in 100 μ L of fresh PBS before lysis, followed by centrifugation to collect the lysate for nucleosome detection. To account for differences in cell concentrations across conditions, nucleosome levels were normalized using the corresponding MTT assay data, and then expressed as fold-change relative to Epo Con.

Immunophenotyping assays

Maturation of erythroid cells was monitored by determination of surface expression of stage-specific markers CD34, CD45, CD71, and glycophorin A (GPA), using fluorescent

dye-conjugated antibodies (Caltag-Invitrogen, Carlsbad, CA) by flow cytometric analysis. Briefly, cells were washed and resuspended in PBS containing 0.5% BSA and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies at 4°C for 25 min. After two washes, cells were resuspended in 300 µL of PBS/0.5% BSA for flow cytometric analysis on a BD FACS Calibur (BD Biosciences, San Jose, CA). A DNA dye 7-amino actinomycin D (7-AAD, eBioscience, San Diego, CA) was added to the stained cells for 10 min before acquisition to measure the percentage of viable cells.

Statistical analyses

Data are presented as means (SEM) of two to five independent experiments performed in duplicate. Differences across treatment groups were tested for statistical significance using ANOVA, while pairwise comparisons were performed by paired and unpaired Student's *t*-tests. Statistical significance for all tests was set at $P < 0.05$.

Results

An in vitro model for studying regulation of erythropoiesis

Studies to determine the molecular mediators and mechanisms involved in the suppression of the erythropoietic response require a reliable ex vivo or in vitro model of erythroid development. We utilized principles described in earlier studies [27,28] to develop an in vitro model of erythropoiesis using peripheral-blood mobilized CD34+ cells. Using a carefully optimized cocktail of growth factors, small quantities of CD34+ cells were first expanded without significant differentiation, and then induced towards erythroid lineage by addition of Epo (Fig. 1). The efficiency and effectiveness of erythropoiesis was continuously monitored during 14 days of erythroid cell growth and development by examining two important parameters: cell proliferation and maturation. Since there is differential expression of surface markers at key developmental stages of erythroid cell maturation (Fig. 2), cell differentiation during secondary culture was monitored using immunophenotypic analyses by multi-color flow cytometry. As CD34+ cells develop through the burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E) stages to more mature erythroblasts and reticulocytes, CD34 and CD45 expression is lost, while expression of CD71 (transferrin receptor) and glycophorin-A (GPA) are gained (Fig. 2A) [31]. Thus, immature CD34+ progenitors on day 3 expressed high levels of CD45 and CD71, but were negative for GPA (Fig. 2B). By day 10 (7 days of Epo stimulation), expression of CD34 was completely lost, CD45 was down-regulated, and a majority of cells expressed GPA, demonstrating erythroid maturation (Fig. 2B, and Table I). Of note, expression of other lineage markers, including CD3, CD14, and HLA-DR was very low or absent (Table I, and data not shown), confirming that the vast majority of the cells were committed erythroid cells by day 10. In contrast, cells cultured in the absence of Epo (No Epo) retained high expression of CD34 and CD45, expressed low levels of GPA, and also expressed HLA-DR (Table I).

Effects of PfHz, PfHz-stimulated PBMC-conditioned media, and inflammatory mediators on erythroid cell proliferation

To examine the role of PfHz and PfHz-induced soluble mediators on erythroid cell development, CD34+ cells were stimulated with Epo in the presence of PfHz (or sHz), or PfHz-stimulated PBMC-conditioned media (i.e., CM-Con, CM-PfHz, and CM-sHz). In addition, the effects of the malaria-associated inflammatory mediators (i.e., TNF- α and NO) on erythropoiesis were also examined. NO was provided in culture by two NO donors, PAPANONOate (half-life 15 min), and DETANONOate (half-life 22 hrs) to demonstrate the effects of transient and prolonged exposure to NO. In the absence of Epo stimulation (No Epo), CD34+ cells failed to thrive and by day 14, cell proliferation was 5% of that observed in cells stimulated with Epo (Epo Con, Fig. 3A). Both PfHz and sHz transiently suppressed Epo-induced erythroid cell proliferation by 15% on day 8, ($P < 0.05$ for all comparisons), but by day 14 there were no statistically significant differences in cell counts compared with cultures stimulated with Epo alone (Fig. 3A). Addition of all PBMC-conditioned media was detrimental to the proliferation of erythroid progenitors during the 14-day culture period. However, the effects of CM-PfHz and CM-sHz were significantly greater than CM-Con and by day 14 erythroid cell proliferation was decreased by 50% in CM-PfHz and CM-sHz compared with 20% in CM-Con ($P < 0.05$ for both comparisons, Fig. 3B). Addition of rhTNF- α exerted a substantial and sustained, dose-dependent suppressive effect on erythroid cell proliferation throughout the culture period, with the effects of TNF- α ranging from a 15% decrease on day 6 to 69% by day 14. These suppressive effects of TNF- α were statistically significant for both doses on days 8, 10, and 14 ($P < 0.05$ for all comparisons, Fig. 3C). Treatment with both PAPANONOate and DETANOate elicited similar patterns of cell proliferation, characterized by a marked early, dose-dependent suppression (13%–30% for PAPANONOate and 45%–95% for DETANOate) of erythroid cell proliferation on days 6 and 8 ($P < 0.05$ for all comparisons, Fig. 3D,E). Although there was a trend towards recovery by days 10 and 14, cell proliferation in the presence of NO donors remained below baseline levels, maintaining statistically significant differences for DETANONOate ($P < 0.05$ for all comparisons, Fig. 3D), but not for PAPANONOate ($P < 0.10$ for all comparisons, Fig. 3E). Taken together, these results demonstrate that PfHz, sHz, PfHz-stimulated PBMC conditioned media, and malaria-associated inflammatory mediators suppress erythroid development, however, these effects differed both temporally and by magnitude.

Effects of PfHz, PfHz-stimulated PBMC-conditioned media, and inflammatory mediators on erythroid cell survival

Erythroid cell apoptosis was measured to determine if the effects of PfHz, CM-PfHz, and inflammatory mediators on proliferation was due to altered cell viability. To ensure detection of total apoptosis, nucleosome concentrations were determined in both cell lysates (early-stage apoptosis) and culture supernatants (late-stage apoptosis) after 3 days of Epo stimulation (i.e., after 6 total days in culture). Although treatment with either PfHz or sHz had no effect on apoptosis, addition of CM-Con, CM-PfHz and CM-sHz significantly increased early- and late-stage apoptosis ($P < 0.05$ for all comparisons, Fig. 4A). In addition, levels of late-stage apoptosis were more than twofold higher in cultures treated with CM-

*Pf*Hz and CM-sHz compared to CM-Con ($P < 0.05$ for both comparisons, Fig. 4A). Treatment with rhTNF- α (10 and 100 ng/mL) had no significant effect on early- or late-stage erythroid cell apoptosis relative to Epo Con levels ($P > 0.05$ for all comparisons, Fig. 4B). While addition of PAPANONOate (50 and 100 μ M) did not significantly affect early-stage apoptosis ($P > 0.05$ for all comparisons), late-stage apoptosis was elevated about threefold in cells cultured in the presence of PAPANONOate relative to Epo Con ($P < 0.05$ for 100 μ M only, Fig. 4C). Treatment of cells with DETANONOate (both 50 and 100 μ M) caused a significant and dose-dependent increase in erythroid cell apoptosis (3-fold to 40-fold over Epo Con, $P < 0.05$ for all comparisons), with the differences being more pronounced for late-stage apoptosis (Fig. 4C). Thus, induction of apoptosis appears to contribute to suppression of erythroid cell proliferation in cultures treated with CM-*Pf*Hz, CM-sHz, and NO donors, while the effects of TNF- α on cell proliferation appear to be independent of apoptosis.

Effects of *Pf*Hz, *Pf*Hz-stimulated PBMC-conditioned media, and inflammatory mediators on differentiation of erythroid progenitors

Suppression of erythropoiesis during malaria results from both ineffective reticulocyte production and dyserythropoiesis [10,11], suggesting that erythroid cell maturation may be impaired. As such, we investigated the impact of *Pf*Hz, CM-*Pf*Hz, TNF- α , and NO on differentiation of erythroid progenitors through immunophenotyping. Treatment of cells with *Pf*Hz or sHz reduced the percentage of cells expressing GPA on day 10 from 87% in Epo-Con to 78% and 79% respectively, and increased retention of surface expression of CD45 from 21% in Epo-Con to 30% and 29% ($P < 0.05$, Fig. 5A and Table I), suggesting inhibition of erythroid maturation. In contrast, the addition of CM-*Pf*Hz or CM-sHz had no significant effect on erythroid maturation relative to treatment with CM-Con ($P > 0.05$ for both comparisons, Fig. 5B and Table I). Treatment with a high dose of TNF- α slightly augmented erythroid cell maturation as evidenced by a down-regulation of CD45 and a marginal increase in GPA expression, however, none of the concentrations of TNF- α yielded significant changes ($P > 0.05$ for all comparisons; Table I and Fig. 5C). Conversely, in the presence of NO donors, GPA expression was reduced from 87% to as low as 70%, and retention of CD45 and CD34 increased from 21% and 1% in Epo Con to as much as 37% and 11% in DETANONOate respectively (Table I, Fig. 5D,E), illustrating significant inhibition of erythroid maturation. The effects of NO were dose-dependent and reached significance for DETANONOate ($P < 0.05$ for all comparisons), but not for PAPANONOate ($P > 0.1$ for all comparisons). Taken together, these data demonstrate that NO is a potent inhibitor of erythroid cell maturation, whereas soluble inflammatory mediators in *Pf*Hz-stimulated PBMC-conditioned media, as well as TNF- α have minimal effects on erythroid cell maturation.

Discussion

The disease burden from malaria falls largely on infants and young children that develop severe life-threatening anemia (i.e., SMA). We have recently shown that one of the primary mechanisms associated with SMA is a decreased erythropoietic response [2,3]. While it is clear that an altered balance of soluble pro- and anti-inflammatory mediators plays an

important role in suppression of erythropoiesis in children with malaria [2,3,14,17,18,26,32–34], the underlying molecular mechanisms that govern this phenomenon are largely undefined. One of the primary challenges that have limited this area of research is the inherent difficulty in obtaining bone marrow aspirates from children with malaria, based on both practical and ethical issues. To overcome this barrier, we built on previously described principles [27,28] to develop an in vitro model system for investigating erythropoiesis by purifying CD34+ cells from PBMCs, pre-expanding the cells using a carefully optimized combination of growth factors that favor proliferation in the absence of differentiation, followed by induction toward erythropoiesis through stimulation with Epo. The novelty of our model system is that it offers an advantage over traditional methods such as microscopy, since the efficiency and effectiveness of erythropoiesis in the in vitro model system utilizes a combination of biochemical and immunophenotyping methods to more quantitatively define erythroid cell survival, proliferation, and maturation. Moreover, this methodology also provides a more objective, rapid, and reproducible strategy for assessing the erythropoietic response compared with traditional methods.

Since our previous studies, as well as those of others, demonstrated that *Pf*Hz accumulation in leukocytes is a strong statistical predictor of SMA and suppression of erythropoiesis [3,8,9,12,13,15,16,18,35–38], we determined the impact of *Pf*Hz, sHz, and conditioned media from PBMC stimulated with *Pf*Hz on erythroid development using the in vitro model system. These experiments revealed that the *Pf*Hz-conditioned media were more potent in suppressing erythropoiesis than the direct addition of *Pf*Hz (or sHz), suggesting that inhibition of erythroid development was largely due to soluble mediators released from PBMCs (i.e., indirect inhibition) rather than accumulation of malarial products within the hematopoietic progenitors [39] (direct inhibition). Although the addition of control conditioned media (i.e., cells not stimulated with malarial products) caused a progressive decline in cell proliferation, the effect of conditioned media from *Pf*Hz- and sHz-treated PBMCs was significantly greater after 14 days. This suggests that phagocytosis of malarial pigment promotes enhanced release of soluble mediators that suppress erythropoiesis beyond what is present in culture media from nonstimulated cells. These findings are consistent with data describing the suppression of erythroid-progenitor growth in bone marrow-derived cell cultures supplemented with *Pf*Hz and supernatants from *Pf*Hz-fed monocytes [40]. However, our data appear to be in contrast to a recent report suggesting that *Pf*Hz-stimulated macrophages were not detrimental to the development of erythroid cells [39]. Additional experiments examining the release of oligonucleosomes revealed that the decrease in erythroid progenitor proliferation in response to conditioned media was associated with the induction of apoptosis. Taken together, these in vitro findings support our hypothesis that decreased erythropoietic responses in children with malaria may, at least in part, be due to *Pf*Hz-induced release of soluble mediators from leukocytes that, in turn, promote induction of cell death in erythroid progenitors.

We have previously shown that TNF- α and NO, two potent proinflammatory soluble mediators, are elevated in the peripheral circulation of children with malarial anemia [17,25,26]. In addition, we have shown that both of these soluble mediators are significantly elevated in vitro following phagocytosis of *Pf*Hz and sHz by leukocytes [17,18]. The effects

of these soluble mediators on erythropoiesis were, therefore, investigated in the in vitro CD34 model. Both TNF- α and NO substantially suppressed erythroid cell proliferation in a dose-dependent manner, with inhibition margins of up to 65% for TNF- α and 90% for NO donors, respectively. These findings are supported by previous studies showing that TNF- α and NO have suppressive effects on erythroid cell development [22,31,41–44]. Results presented here extend this important previous work by showing that NO, but not TNF- α , can suppress erythropoiesis through a mechanism that involves induction of apoptosis. Furthermore, although TNF- α had no significant effects on erythroid cell differentiation, high concentrations of NO donors significantly inhibited the maturation of erythroid cells, characterized by reduced GPA expression. It is important to note that DETANONOate had a more significant impact on both erythroid proliferation and maturation than PAPANONOate. Considering the fact that DETANONOate has a half-life of 22 hrs, while that of PAPANONOate is 15 min., these findings suggest that sustained release of NO in the environment has a more profound effect on erythropoiesis than a short-lived pulse of NO such as that generated by PAPANONOate. Although a recent study found no evidence that malaria-induced inflammatory mediators were responsible for increased apoptosis of Hz-treated erythroid progenitors, those investigations did not examine the role of NO [39]. Based on our previous investigations [17] and the in vitro data presented here, we postulate that phagocytosis of malarial pigment by macrophages in the local bone marrow milieu promotes the sustained release of NO that suppresses erythropoiesis in children with SMA through mechanisms that involve both reduced erythroid survival and maturation.

It is important to note that these experiments relied upon erythropoietic cultures derived from CD34+ cells of healthy US donors, as well as PBMCs derived from malaria-naïve healthy US donors, who do not have the potential repertoire of malaria-specific adaptations that exposed individuals may have. It is thus possible that those children born with the genetic consequence of ancestral malaria exposures may have greater or lesser resistance to these soluble mediators released from *Pf*H₂O₂-stimulated monocytes. It would be interesting to use CD34+ cells and monocytes from a donor group whose regional heritage has indeed been exposed to the malaria parasite.

In summary, the present study describes a novel in vitro model for investigating erythropoiesis based on isolation of CD34+ hematopoietic stem cells from peripheral blood. The use of molecular and biochemical methods for assessing cell proliferation and apoptosis, along with flow cytometric-based phenotypic characterization of erythroid cells, provides a more reliable and objective alternative to exclusive use of microscopic analyses. In addition, we provide evidence that the model developed here offers reproducible results that can be used to study the impact of target soluble mediators implicated in the suppression of erythropoiesis in children with malarial anemia. Use of the in vitro model clearly demonstrated that soluble mediators of inflammation, rather than the potential direct effects of malarial pigment, suppress erythropoiesis through decreased erythroid proliferation and maturation. Although the current study focused on potential mechanisms responsible for suppression of erythropoiesis in children with malarial anemia, this model can be widely used for a variety of diseases and processes to which the erythropoietic cascade plays a central role.

Acknowledgments

Contract grant sponsor: NIH; Contract grant number: R01 AI 51305-01 (to D.J.P.); Contract grant sponsor: Fogarty International Center Training Grant; Contract grant number: D43 TW05884-01 (to D.J.P.).

References

1. World Health Organization. World Malaria Report 2010. WHO Press; Geneva, Switzerland: Available at: http://whqlibdoc.who.int/publications/2010/9789241564106_eng.pdf
2. Were T, Hittner JB, Ouma C, et al. Suppression of RANTES in children with *Plasmodium falciparum* malaria. *Haematologica*. 2006; 91:1396–1399. [PubMed: 17018392]
3. Keller CC, Ouma C, Ouma Y, Awandare GA, et al. Suppression of a novel hematopoietic mediator in children with severe malarial anemia. *Infect Immun*. 2009; 77:3864–3871. [PubMed: 19528216]
4. Burchard GD, Radloff P, Philipps J, et al. Increased erythropoietin production in children with severe malarial anemia. *Am J Trop Med Hyg*. 1995; 53:547–551. [PubMed: 7485716]
5. Burgmann H, Looareesuwan S, Kapiotis S, et al. Serum levels of erythropoietin in acute *Plasmodium falciparum* malaria. *Am J Trop Med Hyg*. 1996; 54:280–283. [PubMed: 8600766]
6. Kurtzhals JA, Rodrigues O, Addae M, et al. Reversible suppression of bone marrow response to erythropoietin in *Plasmodium falciparum* malaria. *Br J Haematol*. 1997; 97:169–174. [PubMed: 9136961]
7. Nussenblatt V, Mukasa G, Metzger A, et al. Anemia and interleukin-10, tumor necrosis factor alpha, and erythropoietin levels among children with acute, uncomplicated *Plasmodium falciparum* malaria. *Clin Diagn Lab Immunol*. 2001; 8:1164–70. [PubMed: 11687458]
8. Casals-Pascual C, Kai O, Cheung JO, et al. Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo. *Blood*. 2006; 108:2569–2577. [PubMed: 16804108]
9. Awandare GA, Ouma Y, Ouma C, et al. Role of monocyte-acquired hemozoin in suppression of macrophage migration inhibitory factor in children with severe malarial anemia. *Infect Immun*. 2007; 75:201–210. [PubMed: 17060471]
10. Wickramasinghe SN, Abdalla SH. Blood and bone marrow changes in malaria. *Baillieres Best Pract Res Clin Haematol*. 2000; 13:277–299. [PubMed: 10942626]
11. Chang KH, Stevenson MM. Malarial anaemia: Mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *Int J Parasitol*. 2004; 34:1501–1516. [PubMed: 15582527]
12. Nguyen PH, Day N, Pram TD, et al. Intraleucocytic malaria pigment and prognosis in severe malaria. *Trans R Soc Trop Med Hyg*. 1995; 89:200–204. [PubMed: 7778149]
13. Luty AJ, Perkins DJ, Lell B, et al. Low interleukin-12 activity in severe *Plasmodium falciparum* malaria. *Infect Immun*. 2000; 68:3909–3915. [PubMed: 10858202]
14. Perkins DJ, Weinberg JB, Kremsner PG. Reduced interleukin-12 and transforming growth factor-beta1 in severe childhood malaria: Relationship of cytokine balance with disease severity. *J Infect Dis*. 2000; 182:988–992. [PubMed: 10950804]
15. Lyke KE, Diallo DA, Dicko A, et al. Association of intraleukocytic *Plasmodium falciparum* malaria pigment with disease severity, clinical manifestations, and prognosis in severe malaria. *Am J Trop Med Hyg*. 2003; 69:253–259. [PubMed: 14628940]
16. Perkins DJ, Moore JM, Otieno J, et al. In vivo acquisition of hemozoin by placental blood mononuclear cells suppresses PGE2, TNF-alpha, and IL-10. *Biochem Biophys Res Commun*. 2003; 311:839–846. [PubMed: 14623257]
17. Keller CC, Kremsner PG, Hittner JB, et al. Elevated nitric oxide production in children with malarial anemia: Hemozoin-induced nitric oxide synthase type 2 transcripts and nitric oxide in blood mononuclear cells. *Infect Immun*. 2004; 72:4868–4873. [PubMed: 15271950]
18. Keller CC, Yamo O, Ouma C, et al. Acquisition of hemozoin by monocytes down-regulates interleukin-12 p40 (IL-12p40) transcripts and circulating IL-12p70 through an IL-10-dependent mechanism: In vivo and in vitro findings in severe malarial anemia. *Infect Immun*. 2006; 74:5249–5260. [PubMed: 16926419]

19. Clark IA, Chaudhri G. Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. *Br J Haematol.* 1988; 70:99–103. [PubMed: 3179231]
20. Kwiatkowski D, Cannon JG, Manogue KR, et al. Tumour necrosis factor production in *Falciparum* malaria and its association with schizont rupture. *Clin Exp Immunol.* 1989; 77:361–366. [PubMed: 2680183]
21. Kwiatkowski D, Hill AV, Sambou I, et al. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet.* 1990; 336:1201–1204. [PubMed: 1978068]
22. Anstey NM, Granger DL, Hassanali M, et al. Nitric oxide, malaria, and anemia: Inverse relationship between nitric oxide production and hemoglobin concentration in asymptomatic, malaria-exposed children. *Am J Trop Med Hyg.* 1999; 61:249–252. [PubMed: 10463675]
23. Jaramillo M, Godbout M, Olivier M. Hemozoin induces macrophage chemokine expression through oxidative stress-dependent and-independent mechanisms. *J Immunol.* 2005; 174:475–484. [PubMed: 15611273]
24. Akman-Anderson L, Olivier M, Luckhart S. Induction of nitric oxide synthase and activation of signaling proteins in *Anopheles* mosquitoes by the malaria pigment, hemozoin. *Infect Immun.* 2007; 75:4012–4019. [PubMed: 17526741]
25. Awandare GA, Hittner JB, Kremsner PG, et al. Decreased circulating macrophage migration inhibitory factor (MIF) protein and blood mononuclear cell MIF transcripts in children with *Plasmodium falciparum* malaria. *Clin Immunol.* 2006; 119:219–225. [PubMed: 16461006]
26. Awandare GA, Goka B, Boeuf P, et al. Increased levels of inflammatory mediators in children with severe *Plasmodium falciparum* malaria with respiratory distress. *J Infect Dis.* 2006; 194:1438–1446. [PubMed: 17054074]
27. Freyssinier JM, Lecoq-Lafon C, Amsellem S, et al. Purification, amplification and characterization of a population of human erythroid progenitors. *Br J Haematol.* 1999; 106:912–922. [PubMed: 10519992]
28. Neildez-Nguyen TM, Wajcman H, Marden MC, et al. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. *Nat Biotechnol.* 2002; 20:467–472. [PubMed: 11981559]
29. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science.* 1976; 193:673–675. [PubMed: 781840]
30. Keller CC, Hittner JB, Nti BK, et al. Reduced peripheral PGE2 biosynthesis in *Plasmodium falciparum* malaria occurs through hemozoin-induced suppression of blood mononuclear cell cyclooxygenase-2 gene expression via an interleukin-10-independent mechanism. *Mol Med.* 2004; 10:45–54. [PubMed: 15502882]
31. Xiao W, Koizumi K, Nishio M, et al. Tumor necrosis factor- α inhibits generation of glycophorin A+ cells by CD34+ cells. *Exp Hematol.* 2002; 30:1238–1247. [PubMed: 12423676]
32. Ouma C, Keller CC, Opondo DA, et al. Association of FCgamma receptor IIA (CD32) polymorphism with malarial anemia and high-density parasitemia in infants and young children. *Am J Trop Med Hyg.* 2006; 74:573–577. [PubMed: 16606986]
33. Ong'echa JM, Remo AM, Kristoff J, et al. Increased circulating interleukin (IL)-23 in children with malarial anemia: In vivo and in vitro relationship with co-regulatory cytokines IL-12 and IL-10. *Clin Immunol.* 2008; 126:211–221. [PubMed: 17945537]
34. Ouma C, Davenport GC, Awandare GA, et al. Polymorphic variability in the interleukin (IL)-1 β promoter conditions susceptibility to severe malarial anemia and functional changes in IL-1 β production. *J Infect Dis.* 2008; 198:1219–1226. [PubMed: 18781863]
35. Schwarzer E, Ludwig P, Valente E, Arese P. 15(S)-hydroxyicosatetraenoic acid (15-HETE), a product of arachidonic acid peroxidation, is an active component of hemozoin toxicity to monocytes. *Parassitologia.* 1999; 41:199–202. [PubMed: 10697856]
36. Schwarzer E, Kuhn H, Valente E, Arese P. Malaria-parasitized erythrocytes and hemozoin nonenzymatically generate large amounts of hydroxy fatty acids that inhibit monocyte functions. *Blood.* 2003; 101:722–728. [PubMed: 12393662]
37. Ochiel DO, Awandare GA, Keller CC, et al. Differential regulation of beta-chemokines in children with *Plasmodium falciparum* malaria. *Infect Immun.* 2005; 73:4190–4197. [PubMed: 15972509]

38. Schwarzer E, Skorokhod OA, Barrera V, Arese P. Hemozoin and the human monocyte—A brief review of their interactions. *Parassitologia*. 2008; 50:143–145. [PubMed: 18693582]
39. Lamikanra AA, Theron M, Kooij TW, Roberts DJ. Hemozoin (malarial pigment) directly promotes apoptosis of erythroid precursors. *PLoS One*. 2009; 4:e8446. [PubMed: 20041181]
40. Giribaldi G, Ulliers D, Schwarzer E, et al. Hemozoin- and 4-hydroxynonenal-mediated inhibition of erythropoiesis. Possible role in malarial dyserythropoiesis and anemia. *Haematologica*. 2004; 89:492–493. [PubMed: 15075084]
41. Johnson RA, Waddelow TA, Caro J, et al. Chronic exposure to tumor necrosis factor in vivo preferentially inhibits erythropoiesis in nude mice. *Blood*. 1989; 74:130–138. [PubMed: 2752104]
42. Shami PJ, Weinberg JB. Differential effects of nitric oxide on erythroid and myeloid colony growth from CD34+ human bone marrow cells. *Blood*. 1996; 87:977–982. [PubMed: 8562969]
43. Rusten LS, Jacobsen SE. Tumor necrosis factor (TNF)-alpha directly inhibits human erythropoiesis in vitro: Role of p55 and p75 TNF receptors. *Blood*. 1995; 85:989–996. [PubMed: 7849320]
44. Cokic VP, Schechter AN. Effects of nitric oxide on red blood cell development and phenotype. *Curr Top Dev Biol*. 2008; 82:169–215. [PubMed: 18282521]

Figure 1.

Experimental design for in vitro model of erythropoiesis. CD34+ hematopoietic stem cells were isolated from donor PBMC by labeling them with magnetic bead-conjugated anti-CD34 monoclonal antibodies followed by positive selection on a magnetic column. Purified CD34+ cells were expanded for 3 days in stem cell growth media containing IL-3, IL-6, and SCF. After 3 days of primary culture, cells were induced toward erythroid lineage development by the addition of erythropoietin alone (Epo Con), or in the presence of *P. falciparum* hemozoin (PfHz), synthetic hemozoin, β -hematin (sHz), PBMC conditioned media: CM-Con (unstimulated PBMC), CM-PfHz (PfHz-stimulated), and CM-sHz (sHz-stimulated); TNF- α , or NO. During 11 days of erythropoiesis, cell proliferation (MTT assay), apoptosis, and maturation (immunophenotyping by flow cytometry) were measured at the indicated time points. An additional control culture was set up without Epo (No Epo).

Figure 2.

Phenotypic markers expressed at key developmental stages of erythroid lineage cells. (A) In response to signals from erythropoietin (Epo), multipotent CD34⁺ stem cells commit to the erythroid lineage, forming burst-forming units (BFU-E). BFU-Es develop into colony-forming units (CFU-E) with the appearance of surface markers including CD71. Under appropriate conditions, including sufficient Epo signals, CFU-Es mature into glycophorin A (GPA)-expressing erythroblasts, accompanied by loss of CD34 and down-regulation of CD45. Following active hemoglobin synthesis, erythroblasts develop into reticulocytes, which mature into erythrocytes accompanied by enucleation and loss of CD71 expression. Markers selected for immunophenotyping in this study are indicated in bold font. (B) Erythroid cell maturation during erythropoiesis shown by immunophenotypic characterization. Surface expression of CD34, CD71, CD45 and GPA was determined by staining with FITC- or PE-conjugated antibodies and analyzed by flow cytometry. Data show expression of surface markers on cultured CD34⁺ cells before addition of Epo (day 3) and 7 days after initiation of Epo stimulation (day 10). Percentages shown are the proportion of total events within the indicated region.

Figure 3.

Effects of PfHz, PfHz-stimulated PBMC-conditioned media, TNF- α , and NO on proliferation of erythroid cells. CD34⁺ stem cells were induced to undergo erythropoiesis by Epo stimulation alone (Epo Con), or in the presence of: (A) *P. falciparum* hemozoin (PfHz) or synthetic hemozoin (sHz), (B) PBMC conditioned media: CM-Con (unstimulated PBMC), CM-PfHz (PfHz-stimulated) and CM-sHz (sHz-stimulated), (C) TNF- α (100 and 10 ng/mL), (D) nitric oxide donor, PAPANONOATE (100 and 50 μ M), and (E) DETANOATE (100 and 50 μ M). As an additional control, some cells were cultured without Epo (No Epo). Cell proliferation was measured at the indicated days using a methylthiazolotetrazolium (MTT)-based assay. Data show cell proliferation of erythroid cells expressed relative to baseline conditions (Epo Con), and are presented as mean (SEM) of three independent experiments. *Time points at which treatments significantly inhibited proliferation relative to the respective controls (PfHz and sHz vs. Epo Con, CM-PfHz and

CM-sHz vs. CM-Con, TNF100 and TNF10 vs. Epo Con, PAPA100 and PAPA50 vs. Epo Con, DETA100 and DETA50 vs. Epo Con, $P < 0.05$, Student's t -test). #Proliferation in No Epo wells were significantly decreased compared to Epo Con on day 6 and remained significant for the entire period of culture in all experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4.

Effects of PfHz, PfHz-stimulated PBMC-conditioned media, TNF- α , and NO on survival of erythroid cells. CD34⁺ stem cells were induced to undergo erythropoiesis by Epo stimulation alone (Epo Con), or in the presence of: (A) *P. falciparum* hemozoin (PfHz) or synthetic hemozoin (sHz), or PBMC conditioned media: CM-Con (unstimulated PBMC), CM-PfHz (PfHz-stimulated) and CM-sHz (sHz-stimulated). (B) TNF- α (100 and 10 ng/mL), and (C) nitric oxide donors, PAPANONOATE (100 and 50 μ M), and DETANOATE (100 and 50 μ M). As an additional control, some cells were cultured without Epo (No Epo). Cellular apoptosis was examined after 3 days of stimulation (day 6 of culture) by measuring the release of nucleosomes in cell lysates (early-stage apoptosis), and supernatants (late-

stage apoptosis) by ELISA. Nucleosome concentrations were expressed as fold-change relative to baseline conditions (Epo Con), and are presented as mean (SEM) of 3 independent experiments. *Treatments that significantly increased apoptosis relative to the respective controls (CM-*Pf*Hz and CM-sHz vs. CM-Con, PAPA100 vs. Epo Con, DETA100, and DETA50 vs. Epo Con, $P < 0.05$, Student's *t*-test). #Both early- and late-stage apoptosis in No Epo wells were significantly increased compared to Epo Con in all experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

Effects of PfHz, PfHz-stimulated PBMC-conditioned media, TNF- α , and NO on maturation of erythroid cells. CD34⁺ stem cells were induced toward erythroid differentiation by Epo stimulation alone (Epo Con), or in the presence of: (A) *P. falciparum* hemozoin (PfHz) or synthetic hemozoin (sHz), (B) PBMC conditioned media: CM-Con (unstimulated PBMC), CM-PfHz (PfHz-stimulated) and CM-sHz (sHz-stimulated), (C) TNF- α (100 ng/mL), (D) nitric oxide donor, PAPANONOATE (100 μ M), and (E) DETANOATE (100 μ M).

Erythroid cell maturation was analyzed by determining surface expression of the erythroid lineage-specific marker glycophorin-A (GPA). Histogram overlays show changes in GPA expression elicited by treatment with the respective mediators relative to baseline control conditions (Epo Con) on day 10 of culture. Cell counts are normalized for each histogram. Data are representative of two to four experiments as summarized in Table I.

TABLE I

Phenotypic Characterization of Erythroid Cell Maturation Status on Day 10

Conditions	Mean (SEM) percent of total live cells expressing markers			
	GPA	CD45	CD34	HLA-DR
Epo Con	87 (4)	21 (2)	1 (0)	0 (0)
<i>Pf</i> Hz	78 (6) *	30 (3) *	1 (0)	0
sHz	79 (6) *	29 (4) *	1 (0)	0
CM-Con	86 (4)	15 (2)	1 (0)	0
CM- <i>Pf</i> Hz	88 (4)	10 (1)	2 (1)	2
CM-sHZ	91 (1)	9 (0)	2 (0)	2
TNF- α (100 ng/mL)	87 (6)	14 (5)	1 (0)	1 (0)
TNF- α (10 ng/mL)	87 (6)	16 (5)	1 (0)	1 (0)
PAPANONOate (100 μ M)	83 (6)	20 (4)	2 (1)	0 (0)
PAPANONOate (50 μ M)	81 (6)	25 (3)	2 (0)	0 (0)
DETANONOate (100 μ M)	70 (4) *	37 (2) *	11 (1) *	0 (0)
DETANONOate (50 μ M)	76 (4) *	29 (2) *	5 (1)	0 (0)
No Epo	16 (5) *	92 (2) *	44 (3) *	44 (3) *

Data shown are proportions of erythroid cells that were positive for the indicated surface markers on day 10, and are presented as mean (SEM) for two to four experiments.

* Significantly different compared with Epo Con ($P < 0.05$, paired t -test).