Plasmodium Products Contribute to Severe Malarial Anemia by Inhibiting Erythropoietin-Induced Proliferation of Erythroid Precursors

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Low reticulocytosis, indicating reduced red blood cell (RBC) output, is an important feature of severe malarial anemia. Evidence supports a role for Plasmodium products, especially hemozoin (Hz), in suppressed erythropoiesis during malaria, but the mechanism(s) involved remains unclear. Here, we demonstrated that low reticulocytosis and suppressed erythropoietin (Epo)–induced erythropoiesis are features of malarial anemia in Plasmodium yoelii– and Plasmodium berghei ANKA–infected mice, similar to our previous observations in Plasmodium chabaudi AS–infected mice. The magnitude of decreases in RBC was a reflection of parasitemia level, but low reticulocytosis was evident despite differences in parasitemia, clinical manifestation, and infection outcome. Schizont extracts and Hz from P. falciparum and P. yoelii and synthetic Hz suppressed Epo-induced proliferation of erythroid precursors in vitro but did not inhibit RBC maturation. To determine whether Hz contributes to malarial anemia, P. yoelii–derived or synthetic Hz was administered to naive mice, and the development of anemia, reticulocytosis, and RBC turnover was determined. Parasite-derived Hz induced significant decreases in RBC and increased RBC turnover with compensatory reticulocytosis, but anemia was not as severe as that in infected mice. Our findings suggest that parasite factors, including Hz, contribute to severe malarial anemia by suppressing Epo-induced proliferation of erythroid precursors.

Keywords. malarial anemia; Plasmodium; reticulocytes; erythropoiesis; mice; hemozoin.

Severe anemia is a life-threatening complication of malaria and is highly prevalent in nonimmune infants and young children in sub-Saharan Africa where Plasmodium falciparum transmission is holoendemic [1]. Anemia is also a common and frequently severe consequence of Plasmodium vivax infection [2]. The cause of malarial anemia is multifactorial and involves hemolysis, erythrophagocytosis, dyserythropoiesis, and ineffective erythropoiesis [3–5]. An important feature of malarial anemia in humans and experimental animals is low reticulocytosis, indicating reduced red blood cell (RBC) output [3–5]. Dyserythropoiesis and suppressed erythropoiesis occur during malaria despite elevated erythropoietin (Epo) levels, but the mechanism(s) is poorly understood.

In vitro and in vivo studies suggest that proinflammatory cytokines, including interferon γ, tumor necrosis factor α (TNF-α), interleukin 12, and macrophage migration inhibitory factor (MIF), as well as Plasmodium products such as hemozoin (Hz), contribute to the pathogenesis of malarial anemia [6–12]. The precise role of parasite products in suppressed erythropoiesis remains unknown. Hz, produced by parasite digestion of hemoglobin, induces macrophages to secrete proinflammatory cytokines and other mediators with inhibitory effects on erythropoiesis [13, 14]. Hz also directly inhibits erythroid progenitor expansion and maturation in vitro, but its contribution to anemia in infected individuals is unknown [8, 10, 14–16]. Although Hz and TNF-α...
synergize to inhibit erythropoiesis in vitro, severe malarial anemia in Kenyan children is independent of serum levels of TNF-α and other cytokines [8, 14]. Rather, the severity of anemia in P. falciparum–infected children correlates with the frequency of Hz-containing blood monocytes [8, 14]. Moreover, there is an association between erythroid and myeloid precursors containing Hz and the presence of abnormal erythroid precursors in the bone marrow of children that die of severe malarial anemia [8].

Mouse malaria models have been useful to delineate the immune responses and cytokines involved in protection and immunopathology and identify novel loci controlling susceptibility to malaria [17–19]. These models have also been used to understand the complex pathogenesis of malarial anemia and to study the roles of RBC clearance, apoptosis of RBC precursors, and parasite virulence in the development of malarial anemia [3, 20–25]. Previously, we demonstrated that blunted Epo-induced growth and maturation of erythroid precursors contribute to suppressed erythropoiesis in Plasmodium chabaudi AS–infected mice [26, 27]. Here, we investigated whether suppressed erythropoiesis contributes to the development of anemia in mice infected with other rodent Plasmodium species. Our findings indicate that severe anemia during lethal infections with Plasmodium yoelii and Plasmodium berghii ANKA are also characterized by low reticulocytosis and suppressed Epo-induced erythropoiesis, responses that are independent of clinical manifestation and infection outcome. We also investigated the contribution of Plasmodium products to suppressed erythropoiesis using in vitro and in vivo approaches. Our findings indicate that parasite factors, including Hz, contribute to erythropoietic suppression, low reticulocytosis, and malarial anemia by inhibiting Epo-induced proliferation of RBC precursors.

**METHODS**

**Mice, Parasites, and Experimental Infections**

Female C57BL/6 (B6) mice aged 8–12 weeks were purchased from Charles River Laboratories. Animal experiments were performed in accordance with the guidelines of the Canadian Council for Animal Care. P. chabaudi AS, P. yoelii 17XL (P. yoelii), and P. berghei ANKA were maintained as described [28–30]. Mice were infected intraperitoneally with 1 × 10⁶ parasitized RBCs (pRBCs). Murine recombinant Epo (10 U/day) was administered intravenously to naive and infected mice for 3 consecutive days as described [7, 27]. Naïve mice were treated intravenously with 700 µg P. yoelii–derived or synthetic Hz, prepared as described below, or administered phosphate-buffered saline (PBS) as a control.

**Parasitological and Hematological Analyses and RBC Turnover**

Parasitemia, reticulocytosis, and hematocrit were determined in infected mice as described [7, 26–28]. The percentage of Hz-containing monocytes was determined on blood smears prepared from buffy coats and stained with Diff-QuikII. Reticulocytosis in Hz-treated mice was assessed daily on blood samples stained with ReticCount according to the manufacturer’s instructions and analyzed by flow cytometry. RBC turnover was assessed in Hz-treated mice by intravenous injection of 100 µl sulfo-N-hydroxysuccinimide-biotin (30 mg/mL) 1 day before Hz treatment [31]. Blood was collected daily, adjusted to 3 × 10⁶ RBC/mL, and labeled with 3 µg/mL phycoerythrin (PE)-streptavidin, and the disappearance of biotinylated RBC was determined by flow cytometry.

**Histology**

Spleens and livers were harvested, fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 5-µm sections. The sections were mounted onto slides and stained with hematoxylin and eosin. Images were photographed using a Zeiss Axioplan2 microscope equipped with Bioquant Nova Prime software (version 6.710.10MT) and acquired and analyzed using Q capture software.

**Preparation of Schizont Extracts and Hz**

Schizont extracts were prepared from blood cultures of P. falciparum (strain 3D7). Briefly, schizont-stage parasites were purified using a 63% Percoll gradient, resuspended in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.12% gentamicin, and subjected to 3 rounds of freeze–thaw followed by sonication [32]. P. yoelii schizont extracts were prepared from infected blood with >90% pRBCs. After lysis with 0.1% saponin, parasites were collected and washed in PBS, and schizont extracts were obtained as described above. Plasmodium–derived Hz was prepared by treating purified schizonts with 2% sodium dodecyl sulfate (SDS) in endotoxin-free PBS followed by sonication for 20 seconds [33]. After centrifugation, the pellet was resuspended in proteinase K (2 mg/mL) and incubated overnight at 37°C. The digested product was washed 3 times with 2% SDS, resuspended in 6 M urea, and incubated on a shaker for 3 hours at room temperature. After 3 washes in 2% SDS and a final PBS wash, the pellet was treated with DNase I (50 U/mL) for 1 hour at 37°C followed by heating to 95°C for 10 minutes to remove excess DNase I. The DNA content of purified Hz was determined using PicoGreen. Synthetic Hz was prepared by the acid-precipitation method from hemin as described [34]. Following vacuum-drying overnight, the material was subjected to infrared spectroscopy and x-ray powder diffraction, respectively, to confirm the identity and crystallinity. Hz preparations were treated with 20 mM sodium hydroxide in 2% SDS for 2 hours at room temperature, and the heme concentration was determined by spectrophotometry [35].

**Thiamphenicol Treatment and K562 Cells**

Naïve mice were administered thiamphenicol (TAP) (15 mg/g body weight) as a subcutaneous implant as described [36] to...
obtain early RBC precursors that respond to Epo in vitro. On days 2–4 postimplant, 100 µL blood were collected. Implants were removed on day 5, and spleen cells were harvested on day 8. To study the effects of parasite products on Epo-induced proliferation, 2 × 10⁵ splenocytes/mL in Iscove’s modified Dulbecco medium (IMDM) supplemented with 10% FCS and 0.12% gentamicin (complete IMDM) with 0.1 µU/mL Epo were seeded into 96-well plates and incubated for 24 hours at 37°C. [3H]-thymidine (specific activity 53.0 Ci/mmol [1.98 TBq/mmol]) was added during the last 2 hours of incubation, and incorporation was determined by scintillation counting. To assess maturation, 1 × 10⁶ splenocytes/mL in complete IMDM with 2 U/mL Epo were cultured for 48 hours and analyzed for TER119 expression by flow cytometry.

The erythroleukemia cell line K562 was maintained in complete IMDM as described [37]. Growth and maturation were assessed by culturing 2 × 10⁵ cells/mL in complete IMDM containing 2.0 mM sodium butyrate for 96 hours and determining [3H]-thymidine incorporation and glycophorin A expression, respectively.

Flow Cytometry

Single cell suspensions of splenocytes were prepared, adjusted to 1 × 10⁶/mL, and FcR blocked with anti-CD16/CD32 monoclonal antibody. Early RBC precursors, which express Epo receptor (EpoR), were identified by staining with rabbit anti-human EpoR antibody followed by fluorescein isothiocyanate (FITC)-conjugated, antirabbit immunoglobulin G antibody [7, 27]. Erythroblasts and transferrin receptor (TfR1)–expressing cells were identified, respectively, by staining with PE-conjugated, anti-TER119 or FITC-conjugated, anti-CD71 monoclonal antibodies [7, 27]. To assess viability, splenocytes were stained with 7-aminoactinomycin D (7-AAD) according to the manufacturer’s instructions. Glycophorin A expression on K562 cells was determined by staining with PE-labeled, anti-glycophorin A monoclonal antibody [37]. Cells were acquired using a FACSCalibur, and data were analyzed using CellQuest Pro software.

Statistical Analyses

Data are presented as mean ± standard error of the mean (SEM). The statistical significance of differences between groups was analyzed by 2-tailed, unpaired Student’s t test. Multiple comparisons were analyzed by analysis of variance. R² was computed for simple linear regression analysis. Statistical analyses were performed using GraphPad Prism (v5.0). Significance was defined as P < .05.

RESULTS

Anemia and Low Reticulocytosis During Nonlethal and Lethal Malaria Infections

Parasitemia, anemia, and reticulocytosis were compared in B6 mice for 7–8 days after infection with P. chabaudi AS, P. yoelii, or P. berghei ANKA. As previously observed, P. chabaudi AS

Figure 1. Course of parasitemia, anemia, and reticulocytosis in Plasmodium-infected mice. 86 mice were infected intraperitoneally with 1 × 10⁶ Plasmodium chabaudi AS (A), Plasmodium yoelii (B), or Plasmodium berghei ANKA (C) parasitized red blood cells (pRBCs). Parasitemia, RBC numbers, and percentage reticulocytes in peripheral blood were determined daily until days 7–8 postinfection when P. yoelii– and P. berghei ANKA–infected mice died. The course of parasitemia is presented as mean percentage pRBC in the inset for each infection. Data for RBC and reticulocytes are presented as mean ± standard error of the mean for 4 mice per group. *P < .05, naive (day 0) vs infected mice. Similar results were obtained in 3 independent experiments. Abbreviations: P. berghei, Plasmodium berghei; P. chabaudi, Plasmodium chabaudi; P. yoelii, Plasmodium yoelii; RBC, red blood cell.
infection in resistant B6 mice resulted in a peak parasitemia of 30%–35% pRBC on day 8 postinfection (Figure 1A); thereafter, parasitemia decreased, and the infection was eliminated by 3–4 weeks [7, 20, 26]. Coincident with parasite replication on days 6–8 postinfection, anemia developed with significantly fewer RBCs and reticulocytes in infected mice compared with naive mice [7, 26]. P. yoelii infection was fatal to 100% of B6 mice by day 7 postinfection, when the mice succumbed with high parasitemia (>90% pRBCs) (Figure 1B). Severe anemia developed, and there were few reticulocytes evident in the peripheral blood. As previously shown, 100% of B6 mice succumbed to P. berghei ANKA infection by 7–8 days postinfection with neurological symptoms characteristic of experimental cerebral malaria [20]. Despite low parasitemia (<10% pRBCs), P. berghei ANKA–infected mice had significantly lower than normal levels of RBC and reticulocytes (Figure 1C). These observations indicate that severe anemia and low reticulocytosis are features of blood-stage malaria in Plasmodium–infected mice despite differences in clinical manifestation and infection outcome.

Suppressed Erythropoiesis Contributes to Low Reticulocytosis in Plasmodium–Infected Mice

To determine whether suppressed erythropoiesis contributes to low reticulocytosis in mice infected with P. yoelii or P. berghei ANKA, we assessed Epo-induced growth and maturation of splenic erythroid precursors from naive and infected mice, either untreated or treated with Epo. Because significant differences in spleen weight and cellularity were apparent among the various mouse groups (Supplementary Table 1), the frequency rather than the absolute number of each cell type is presented (Figure 2). Consistent with our observations in P. chabaudi AS–infected mice, the frequency of splenic EpoR⁺ cells was significantly higher in P. yoelii– and P. berghei ANKA–infected mice compared with naive mice and after Epo treatment of infected and naive mice (Figure 2A) [7, 27]. In response to Epo stimulation in vitro, splenocyte proliferation was significantly lower in Epo-treated infected mice compared with cells from similarly treated naive mice (Figure 2B). There were significant increases in the frequencies of TER119⁺ erythroblasts in P.
**In Vitro Effects of Parasite Products on Erythropoiesis**

Next, we investigated whether parasite products suppress RBC growth and maturation in vitro using splenocytes from TAP-treated mice and K562 cells as responder cells. *P. falciparum* and *P. yoelii* schizont extracts significantly suppressed splenocyte proliferation in a dose-dependent manner (Figure 3A), and the inhibitory effects were Epo-dependent (data not shown). *P. falciparum* schizont extracts also significantly inhibited sodium butyrate-induced K562 cell proliferation (Figure 3B). Exposure to *P. falciparum* and *P. yoelii*–derived Hz resulted in significant decreases in Epo-induced splenocyte proliferation with >70% inhibition at 10 µg/mL (Figure 3C). Synthetic Hz significantly suppressed Epo-induced proliferation, albeit to a lesser extent than parasite-derived Hz. Neither *Plasmodium*–derived nor synthetic Hz affected the viability of splenocytes from TAP-treated mice as assessed by 7-aminoactinomycin D (7-AAD) staining (data not shown).

Consistent with previous studies, approximately 40% of splenocytes from TAP-treated mice expressed TER119 after Epo stimulation, indicating maturation of EpoR+ cells to erythroblasts, compared with <5% of medium control cells (Figure 4A) [36]. *P. falciparum* and *P. yoelii* schizont extracts induced erythroid cell maturation in the absence of Epo, and *P. falciparum* schizont extracts significantly increased Epo-induced maturation. *P. falciparum* schizont extracts induced significantly higher glycophorin A expression on K562 cells than sodium butyrate alone, indicating maturation (Figure 4B). Neither *Plasmodium*–derived Hz nor synthetic Hz altered erythroid precursor maturation in the presence or absence of Epo (Figure 4C).

**Hz Accumulation in Tissue Macrophages and Blood Monocytes During Malaria Infection**

Histological analyses of tissues harvested from infected mice on days 7–8 postinfection showed marked expansion of the splenic chabaudi AS– and *P. berghei* ANKA–infected mice compared with naive mice (Figure 2C). After Epo treatment, *Plasmodium*–infected mice had >50% decreases in the frequencies of TER119+ erythroblasts compared with naive mice regardless of the infecting parasite species. These data indicate that blunted Epo-induced erythropoiesis contributes to anemia during *P. yoelii* and *P. berghei* ANKA infections similar to *P. chabaudi* AS infection.

We also examined the effects of different parasite infections on CD71 (TfR1) expression. In naive mice, Epo treatment induced a significantly higher frequency of CD71+ splenocytes compared with untreated mice (P < .0001; Figure 2D). There was a significant increase in CD71+ cell frequency during *P. chabaudi* AS infection compared with naive mice, but this cell population remained unchanged compared with naive mice after Epo treatment. *P. yoelii*– and *P. berghei* ANKA–infected mice, with or without Epo treatment, had low frequencies of CD71+ cells regardless of Epo treatment, suggesting that splenocyte CD71 expression varied during infection with different *Plasmodium* parasites.

![Figure 3. Inhibition of erythroid cell growth by schizont extracts and hemoglobin (Hz). Single cell suspensions of spleen cells from thiamphenicol-treated B6 mice were prepared, and 2 × 10⁵ splenocytes/mL were cultured for 24 hours in complete Iscove’s modified Dulbecco medium (IMDM) containing 2.0 mM sodium butyrate and the indicated amounts of *Plasmodium falciparum* or *Plasmodium yoelii* schizont extract (0.6–5 × 10⁹ schizont equivalents/mL) (A) or *Plasmodium*-derived or synthetic Hz (10–75 µg/mL) (C). Proliferation was determined by [³H]-thymidine incorporation, and the percentage inhibition was calculated as follows: sodium butyrate cpm – schizont extract cpm/sodium butyrate cpm × 100. B, The data are presented as mean ± SEM. *P<.05, medium vs schizont extract. Similar results were obtained in 3 independent experiments. Abbreviations: Epo, erythropoietin; Hz, hemoglobin; *P. falciparum*, *Plasmodium falciparum*; *P. yoelii*, *Plasmodium yoelii*.](image)
Maturation of erythroid cells is not inhibited by schizont extracts or hemozoin (Hz). Single cell suspensions of spleen cells from thiamphenicol-treated B6 mice were prepared, and 1 × 10⁶ splenocytes/mL were cultured for 48 hours in complete Iscove’s modified Dulbecco medium (IMDM) containing 2.0 U/mL erythropoietin (Epo) and the indicated concentrations of Plasmodium falciparum or Plasmodium yoelii schizont extract (5 × 10⁷ schizont equivalents/mL) or Plasmodium-derived or synthetic Hz (75 µg/mL) (C). Maturation of EpoR⁺ cells to erythroblasts was determined based on TER119 expression by flow cytometry. Because none of the doses tested for P. falciparum or P. yoelii schizont extract (0.6–5 × 10⁷ schizont equivalents/mL) and Hz (10–75 µg/mL) significantly suppressed maturation, only the results of the highest doses are shown. Data are presented as mean frequency of TER119⁺ cells ± standard error of the mean (SEM). *P < .05, medium vs schizont extract or Hz.

Figure 5. The percentage of hemozoin (Hz)–containing blood monocytes correlates with the severity of anemia during Plasmodium chabaudi AS infection. B6 mice (n = 5) were infected intraperitoneally with 1 × 10⁶ P. chabaudi AS parasitized red blood cells. Blood was collected on the indicated days postinfection, and parasitemia, Hz-containing monocytes, and hematocrit were determined. A, Course of parasitemia in P. chabaudi AS–infected mice. Data are presented as mean ± standard error of the mean (SEM). *P < .05, medium vs schizont extract. B, The number of Hz⁺ cells per 100 monocytes during P. chabaudi AS infection. The percentage of Hz⁺ monocytes was determined on blood smears prepared from Buffy coats. Data are presented as mean ± SEM; *P < .05 vs day 0. C, Association between Hz-containing blood monocytes and hematocrit in P. chabaudi AS–infected mice. Linear regression analysis of mean percentage hematocrit and the mean percentage of Hz⁺ monocytes; *P < .005. Similar results were obtained in a replicate experiment. Abbreviations: Hz, hemozoin; ND, not detected.
white pulp with an increase in periarteriolar lymphoid sheaths to approximately twice the size in infected mice compared with naive mice (Supplementary Figure 1). Expansion of the red pulp indicative of erythropoiesis was evident in the spleens of infected mice. Mice infected with *P. chabaudi* AS and especially those infected with *P. yoelii* exhibited abundant Hz accumulation in red pulp macrophages, consistent with high blood parasitemia, whereas *P. berghei* ANKA–infected mice exhibited mild pigmentation in red pulp macrophages. Erythrophagocytosis was also evident in the spleens of infected mice. Analysis of liver sections from *P. chabaudi* AS–, *P. yoelii–*, and *P. berghei* ANKA–infected mice demonstrated Hz accumulation in Kupffer cells (data not shown).

To investigate the relationship between Hz-containing phagocytes and the severity of malarial anemia, we determined the percentage of Hz+ monocytes in *P. chabaudi* AS–infected mice (Supplementary Figure 2). The frequency of Hz+ monocytes peaked on day 10 postinfection around the time of peak parasitemia (Figure 5A and 5B). Hz remained evident in monocytes through 24 days postinfection when pRBC were no longer visible in peripheral blood. The percentage of Hz+ monocytes correlated significantly with the hematocrit (*P* < .005; Figure 5C), but not with reticulocytosis (*P* = .41; data not shown).

**P. yoelii–Derived Hz Induces Anemia and Increases RBC Turnover In Vivo**

To determine whether Hz induces anemia in vivo, naive mice were treated with *P. yoelii–*derived or synthetic Hz by intravenous administration of 700 μg, equivalent to 30 μM heme/kg or 10% parasitemia in *P. falciparum*–infected adults [38]. There were no significant decreases in RBC in mice treated with synthetic Hz compared with PBS control mice through 50 days, although there were small but significant increases in reticulocytes on days 2 and 3 after treatment (Figure 6A and 6C). RBC numbers decreased significantly in mice treated with *P. yoelii–* derived Hz on days 1–6 after treatment with compensatory reticulocytosis evident on days 4–7 with a peak on day 5 (mean reticulocytes ± SEM, 12.1% ± 0.4%) (Figure 6B and 6D). RBC turnover was similar in mice treated with synthetic Hz or PBS. In contrast, mice treated with *P. yoelii–* derived Hz exhibited significantly increased RBC turnover compared with PBS control. Together, these data indicate that *Plasmodium*–derived Hz contributed to anemia during blood-stage malaria.

**DISCUSSION**

Suppressed erythropoiesis is an important factor underlying low reticulocytosis and an inability to replenish RBCs lost by hemolysis and other mechanisms during malaria. Consistent with our previous findings in *P. chabaudi* AS–infected mice, we observed that low reticulocytosis and suppressed Epo-induced erythropoiesis are features of malarial anemia in *P. yoelii–* and *P. berghei* ANKA–infected mice [7, 26, 27]. Infection with these parasites induced anemia and decreased RBCs despite differences in clinical manifestation and infection outcome. A recent study in mice infected with different *P. chabaudi* clones revealed a strong correlation between parasitemia level and decreased RBC numbers [22]. Here, we observed that *P. yoelii–* infected mice had >90% pRBCs with >70% RBC decrease, *P. chabaudi* AS–infected mice had 30%–35% pRBCs and an approximate 60% RBC decrease, and *P. berghei* ANKA–infected mice had <10% pRBCs with a 20% RBC decrease. In addition, reticulocytosis was uniformly low, especially in *P. berghei* ANKA–infected mice.

Studies in *P. falciparum–* and *P. vivax–infected individuals support a role for suppressed erythropoiesis in malarial anemia [2–5]. We previously showed that suppressed erythropoiesis in *P. chabaudi* AS–infected mice is due to blunted Epo-induced growth and maturation of erythroid precursors [7, 27]. RBC precursors from *P. yoelii–* and *P. berghei* ANKA–infected mice had significantly decreased Epo-induced proliferation in vitro. This was despite significant increases in the frequencies of EpoR+ cells compared with naive mice and higher frequencies of EpoR+ cells in Epo-treated infected mice compared with similarly treated naive mice. There were significantly higher frequencies of TER119+ erythroblasts in *P. chabaudi* AS– and *P. berghei* ANKA–infected mice but not *P. yoelii–*infected mice compared with naive mice. The frequencies of TER119+ erythroblasts were significantly lower in infected mice compared with naive mice after Epo treatment regardless of the *Plasmodium* species. Together, these observations suggest that decreased growth and maturation of EpoR+ cells to TER119+ erythroblasts may be a common mechanism underlying suppressed erythropoiesis during malaria but that the magnitude may vary with the infection.

As we previously observed, there were significantly higher frequencies of CD71+ spleen cells in *P. chabaudi* AS–infected mice compared with naive mice, and the frequency of CD71+ splenocytes was similar in *P. chabaudi* AS–infected mice and naive mice treated with Epo [7, 27]. Surprisingly, CD71+ splenocyte frequencies were similarly low in *P. berghei* ANKA– and *P. yoelii–*infected mice and naive mice; CD71+ expressing cells remained low in *P. yoelii–* and *P. berghei* ANKA–infected mice after Epo treatment. Expression of CD71 or TfR1, essential for uptake of iron bound to transferrin, is normally increased on maturing erythroblasts that require iron for hemoglobin synthesis as well as on other actively growing cells [39]. During *P. chabaudi* AS infection, CD71 expressing erythroblasts decrease concomitant with expanding populations of activated CD71+ leukocytes [7, 27]. These findings suggest that impaired erythroblast maturation may be a consequence of decreased iron uptake by developing RBCs and that the decrease may vary with the infection, but additional studies are required to address these possibilities.
Although *Plasmodium* products, especially Hz, induce macrophages to secrete proinflammatory cytokines that inhibit erythropoiesis, these products directly inhibit developing RBCs, possibly by interfering with cell-cycle regulation with decreased expression of GATA-1 and inhibition of receptors required for erythroid cell growth and maturation, including EpoR and...
CD71 [3–6, 8, 10, 14–16]. Similar to direct inhibitory effects of *P. falciparum* and *P. vivax* pRBCs on RBC growth in vitro, we observed that schizont extracts from *P. falciparum* and *P. yoelii* suppressed Epo-induced proliferation of murine EpoR+ cells and sodium butyrate–induced K562 cell proliferation [8, 40]. Although previous studies demonstrated that parasite products inhibit Epo-induced maturation of human CD34+ cells, we found no evidence that RBC maturation was decreased by schizont extracts [8, 14–16]. Consistent with previous observations, *Plasmodium*–derived Hz suppressed proliferation but not maturation [8]. Synthetic Hz also had growth inhibitory effects, but the effects of *Plasmodium*–derived Hz were >2-fold higher than those of synthetic Hz. This difference may be related to differences in crystal size between natural and synthetic Hz [41]. In addition, we observed that Epo–induced proliferation of splenocytes from TAP-treated mice was suppressed to a greater degree at equal concentrations of *P. falciparum*–derived Hz than *P. yoelii*–derived Hz. This finding suggests that Hz from various *Plasmodium* species may suppress RBC growth to different degrees and may partially explain why infections with different parasites cause anemia of similar severity despite differences in parasitemia levels, as observed here and previously [22, 26].

The ability of Hz to directly inhibit erythropoiesis in vitro suggests that it may contribute to malarial anemia in vivo [8, 10, 14–16]. Earlier studies revealed the presence of Hz in bone marrow macrophages in *P. falciparum*–infected patients with anemia [42], and there is a strong correlation between the frequency of Hz-containing blood monocytes and the severity of anemia in *P. falciparum*–infected children [8, 14]. We observed that the frequency of Hz+ monocytes correlated significantly with the severity of anemia in *P. chabaudi* AS–infected mice. We also provide important evidence that parasite-derived Hz induced anemia in vivo with decreased RBCs, increased RBC turnover, and compensatory reticulocytosis. These findings are consistent with a previous study demonstrating that administration of soluble *P. chabaudi* AS– or *P. berghei* ANKA–derived antigens induces anemia, albeit less severe than that induced by infection [9]. Hz alone may thus not account for the severity of malarial anemia, but may synergize with other parasite factors or mediators such as cytokines to induce severe anemia [8]. We also observed that *P. falciparum*–derived glycosylphosphatidylinositol inhibited sodium butyrate–induced K562 cell maturation (unpublished observations). Although GPI may attach to the membrane of uninfected RBC and become a target for circulating antiglycosylphosphatidylinositol antibodies, resulting in immune-mediated hemolysis, the ability of glycosylphosphatidylinositol to cause anemia in vivo has not been investigated [43].

In conclusion, our findings suggest that *Plasmodium* products, especially Hz, contribute to suppressed erythropoiesis, low reticulocytosis, and malarial anemia by inhibiting the proliferation of erythroid precursors. Together, these data help to clarify the complexity of suppressed Epo-induced erythropoiesis during malaria, thereby adding to our understanding of malarial anemia. Such understanding is imperative for identifying therapeutic targets and interventions for use in severe cases of malarial anemia.

**Supplementary Data**

Final supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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