Severe malaria, a leading cause of mortality among children and nonimmune adults, is a multisystemic disorder characterized by complex clinical syndromes that are mechanistically poorly understood. The interplay of various parasite and host factors is critical in the pathophysiology of severe malaria. However, knowledge regarding the pathophysiological mechanisms and pathways leading to the multisystemic disorders of severe malaria in humans is limited. Here, we systematically investigate infections with Plasmodium coatneyi, a simian malaria parasite that closely mimics the biological characteristics of P. falciparum, and develop baseline data and protocols for studying erythrocyte turnover and severe malaria in greater depth. We show that rhesus macaques (Macaca mulatta) experimentally infected with P. coatneyi develop anemia, coagulopathy, and renal and metabolic dysfunction. The clinical course of acute infections required suppressive antimalaria chemotherapy, fluid support, and whole-blood transfusion, mimicking the standard of care for the management of severe malaria cases in humans. Subsequent infections in the same animals progressed with a mild illness in comparison, suggesting that immunity played a role in reducing the severity of the disease. Our results demonstrate that P. coatneyi infection in rhesus macaques can serve as a highly relevant model to investigate the physiological pathways and molecular mechanisms of malaria pathogenesis in naive and immune individuals. Together with high-throughput postgenomic technologies, such investigations hold promise for the identification of new clinical interventions and adjunctive therapies.
of cerebral malaria and severe anemia have been studied and the immune-pathological pathways of causation dissected extensively. However, new effective clinical interventions have not come from this body of work, and it has been questioned if these rodent malaria models are relevant to human malaria (7, 8). Non-human primate (NHP) models of malaria infection are available, although certainly underutilized, to study malaria disease states (8–10). The potential of infections of various simian malaria parasites in macaques to identify mechanisms of pathogenesis common to human malaria has been studied to a limited extent (8–10). *P. coatneyi* and *P. falciparum*, although not particularly close phylogenetically, do closely share a number of nearly identical phenotypic characteristics. Both species are characterized by a 48-hour intraerythrocytic developmental cycle, electron-dense knob protrusions on the surface of their infected host cells, and profound deep vasculature sequestration of trophozoite- and schizont-infected erythrocytes. The primary sites of infected erythrocyte adhesion to endothelium for *P. coatneyi* were in the vessels of the heart, fatty tissues, omentum, intestines, and musculature, very similar to tissue sites for *P. falciparum* in human malaria (11–13). In this regard, infection with *P. coatneyi* has been proposed as an optimal primate model for human cerebral malaria (13–16). Although these particular studies have shown histological findings with sequestration of infected erythrocytes in cerebral vasculature, the multisystemic clinical data collected during the course of infection have been sparse or nonexistent. Similarly, compartmentalization of *P. coatneyi*-infected erythrocytes in the placenta in experimentally infected macaques has suggested that *P. falciparum* and *P. coatneyi* may share molecular mechanisms of pathogenesis during pregnancy (17–19). However, as noted above, only limited clinical information is available from these studies. Nevertheless, studies conducted after the discovery of *P. coatneyi* have reported signs of severe disease in the majority of animals and a mortality of 30% to 35% in rhesus monkeys if not treated (20).

We report here a comprehensive characterization of the clinical outcomes of rhesus macaques experimentally infected with virulent stocks of *P. coatneyi*-infected RBCs. We exposed the animals to sequential experimental infections to observe the development of antiparasite immunity and the associated consequences with regard to the altered course of pathogenesis. Subcutaneous antimalarial treatment was required with the first infection to avoid deaths during the development of life-threatening conditions during the acute course of the infections. All malaria-naive rhesus macaques infected in this study developed severe malaria with signs of multisystemic involvement. Hematologic disorders and metabolic dysfunctions including severe anemia and coagulopathy were the most common complications. Both accelerated turnover of uninfected erythrocytes and ineffective erythropoiesis accounted for severe anemia in the course of the initial infection. In stark contrast, only mild anemia and minor metabolic changes were observed in these animals during their subsequent infection, 9 months later. Our results support the use of *P. coatneyi* infections in rhesus macaques as a robust host-parasite model to identify physiological and immunological mechanisms associated with severe malaria pathogenesis relevant to human disease.

**MATERIALS AND METHODS**

**Study design and experimental infections.** Twenty-three male rhesus macaques (*Macaca mulatta*) that had been born and raised at the Yerkes National Primate Research Center were assigned to this study. Procedures used were approved by Emory University’s Institutional Animal Care and Use Committee and followed accordingly. The Hackeri strain of *P. coatneyi* used in these studies came from archived stocks maintained by cryopreservation at the Centers for Disease Control and Prevention (21). Twenty animals were randomized into four groups of 5 animals; and two of these groups were exposed to *P. coatneyi* at two different time points. Ten animals were selected as controls to characterize normal hematologic parameters. Three macaques were infected with *P. coatneyi*-infected erythrocytes from a cryopreserved stabile to serve as donors to obtain freshly drawn infected blood for inoculation of the test animals with a comparable number of viable parasites. Each recipient was inoculated with 2 × 10⁸ *P. coatneyi*-infected erythrocytes/kg of body weight and followed up daily for 30 days. Based on our preliminary data, we expected to have detectable parasitemia in malaria-naïve individuals on day 3 and peaks of parasitemia within 2 weeks after experimental infection. Following subcutaneous antimalarial treatment with a fast-acting drug (artemether), episodes of recrudescence were also expected within 2 weeks after the intervention. To test the effect of antimalarial acquired immunity on disease progression, the animals were exposed to a second experimental infection using the same number of parasites/kg 9 months after the first infection had been cleared. In the course of each infection, the animals were monitored daily for clinical symptoms including anorexia, respiratory distress, and impaired consciousness.

To be able to quantitatively assess the clinical severity daily and facilitate comparison between groups, we developed a rhesus physiological score system (RPSS) (Table 1). Tachypnea was defined as increase in respiratory rate over 20 breaths per minute. Dyspnea was defined as shortness of breath accompanied by intercostal and supraclavicular retractions, nasal flaring, and paradoxical abdominal respiration as previously defined (22). Capillary blood samples were obtained every day by standardized ear prick procedures and collected into EDTA-coated capillary tubes. Blood samples were used to determine hemoglobin concentration using a HemoCue photometer (HemoCue Inc., Lake Forest, CA) and to quantify the parasite load using Giemsa-stained thin and thick smears. Reticulocyte enumeration was achieved by manual counting of new methylene blue-stained reticulocytes in blood smears. Serum levels of fibrinogen degradation products were determined using a direct latex agglutination assay (Pacific Hemostasis, Middletown, VA). On indicated dates, venous blood samples were obtained for blood coagulation tests and blood chemistry analyses. Blood platelet counts were obtained by manual quantification using Giemsa-stained thin smears. To avoid life-threatening clinical conditions, animals with low hemoglobin levels or respiratory distress received subcutaneous treatment with artemether at 2 mg/kg in a single intramuscular injection. The animals were followed up for 30 days and received a curative antimalarial regimen of 4 mg/kg of artemether once, followed by 2 mg/kg/day for 6 days.

<table>
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<tr>
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<tr>
<td>Respiratory rate</td>
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<td>Activity</td>
<td>Good</td>
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<tr>
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<tr>
<td>Platelets (no. of cells/μl)</td>
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<td>Parasitemia* (no. of parasites/μl)</td>
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*For parasitemia levels, additional scores were assigned: 3, 10,000 to 20,000/μl; 4, 20,000 to 40,000/μl; 5, 40,000 to 60,000/μl; 6, 60,000 to 80,000/μl; 7, 80,000 to 100,000/μl; 8, >100,000/μl.*

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in vivo biotinylation. In vivo biotinylation was conducted by intravenous infusion of EZ-Link Sulfo-NHS-biotin (Pierce, Rockford, IL) at 37.5 or 20 mg/kg in phosphate-buffered saline (PBS). The procedure was conducted under anesthesia using a Buretrol infusion set (Baxter Healthcare Corp., Deerfield, IL). To avoid potential anaphylactic reaction, the animals were premedicated with diphenylhydramine at 1 mg/kg. The biotin suspension was infused for 20 min at 40 to 50 microdrops/minute. The system was then flushed using sterile saline solution. The same intravenous line was used for parasite inoculation of the macaques included in the experimental groups. After infusion, the intravenous cannula was removed and blood samples were collected to determine the biotinylation efficacy using flow cytometry.

Characterization of the erythrocyte life span. EDTA capillary blood samples were obtained daily using BD Microtainer capillary blood tubes (Becton, Dickinson, Franklin Lakes, NJ). The cells were washed with PBS and incubated at 3% hematocrit with streptavidin-Alexa Fluor 488 (Invitrogen, Carlsbad, CA) at room temperature in the dark for 30 min. After incubation, the cells were washed twice, resuspended in PBS, and analyzed by flow cytometry using a BD FACSDiVa LSR II (BD Biosciences, San Jose, CA). The percentage of biotinylated cells was calculated as the ratio of positive cells to all erythrocytes. Results are presented as absolute erythrocyte numbers by normalization with erythrocyte counts determined every day in the course of the follow-up period.

Bone marrow core biopsy specimens. Bone marrow core biopsy specimens were obtained using a 13-gauge Jamshidi biopsy needle and standard technique. The biopsy specimens were taken from the femur via the trochanteric fossa or iliac crest. Biopsy imprints were made by gently touching the core on slides. The cores were then fixed in 10% formalin (23) and decalcified in RDO Gold (Apex Engineering Products Corp., Aurora, IL). The specimens were embedded in paraffin and cut at 6 μm.

Analysis of plasma cytokine concentration. Interleukin-1 beta (IL-1β), IL-6, IL-8, macrophage inflammatory protein-1 alpha (MIP1α)/chemokine ligand 3 (CCL3), gamma interferon (IFN-γ), monocyte chemotactic protein-1 alpha (MCP1)/CCL2, macrophage inflammatory protein-1 beta (MIP1β)/CCL4, tumor necrosis factor alpha (TNF-α), metalloproteinase-3 (MMP3), MMP9, RANTES (regulated on activation normal T cell expressed), tissue inhibitory of matrix metalloproteinase-1 (TIMP1), TIMP2, MMP2, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL6R (IL-6 receptor), macrophage-derived chemokine (MDC), and neutrophil-activating protein-2 (NAP2) levels were determined using multiplex luminiscence beads by Aushon Biosystems (Billerica, MA) using primate-optimized reagents. IL-10 and erythropoietin (EPO) were assayed by sandwich enzyme-linked immunosorbent assay (ELISA) using commercial immunoassays according to the manufacturer’s instructions (R&D Systems, Inc., Minneapolis, MN).

Statistical analysis. Statistical analyses were made using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). For assessment of clinical severity and kinetics of circulating biotinylated cells, differences between groups were evaluated by comparing the mean area under the curve (AUC) values using the Mann-Whitney test. Pearson correlation coefficient analysis was performed to determine the association between hemozoin-containing leukocytes, hemoglobin, and parasitemia. Differences in clinical laboratory parameters, coagulation tests, and proinflammatory mediators were evaluated using one-way between-groups analysis of variance (ANOVA) with post hoc Bonferroni’s multiple-comparison posttest. P values of <0.05 were considered significant.

RESULTS

Plasmodium coatneyi infection of naïve rhesus macaques induces severe anemia and a delay in erythroid compensatory mechanisms. P. coatneyi infections were comprehensively monitored in a group of five naïve rhesus macaques. We designed experimental protocols to record clinical and clinical laboratory parameters in the course of the infections. The animals were experimentally challenged intravenously with 1 × 10⁹ P. coatneyi-infected erythrocytes/kg of body weight obtained from an active infection in a donor monkey. To ensure that these infections mimicked the natural course of P. coatneyi infections in rhesus macaques as originally described (20, 21), parasites were inoculated from the original line of P. coatneyi (24), archived as cryopreserved stocks at the CDC and confirmed to have no history of experimental passage in splenectomized animals. Parasitemias, hemoglobin levels, and reticuloocyte counts were determined daily from peripheral blood capillary samples as described previously (25). Similar capillary samples were also taken and monitored from uninfected control animals. Infected erythrocytes were first identified in peripheral blood smears 72 h after experimental challenge (Fig. 1). Parasitemia in the five monkeys followed the expected pattern of peaks on alternating days followed by a sharp drop in parasite levels, coinciding with the maturation and sequestration of the parasites, comparable to that of P. falciparum (21); presumably via predicted variant antigens analogous to the var gene-encoded erythrocyte membrane protein-1 ( PfEMP1) and SICA antigens of P. knowlesi (references 26 and 27 and unpublished data.). Parasite increases were recorded on days 3, 5, and 7 or 9 after experimental infection. The parasitemias peaked on day 9 or 11 postinfection with mean levels of >200,000 parasites/μl. To avoid the onset of life-threatening conditions, the animals received a subcutaneous dose of artemether (2 mg/kg) between days 9 and 11. The subcutaneous antimalaria treatments resulted in a sustained drop in parasitemia, which started to increase again by day 16 (Fig. 1).
The kinetics of hemoglobin concentration showed a steady decrease from the baseline levels and a minimum mean value of 6.4 g/dl on day 11. Two animals developed severe anemia by day 11 with hemoglobin levels lower than 5 g/dl. In one of these subjects, the severe anemia was associated with thrombocytopenia and coagulopathy, complications that ultimately led to disseminated intravascular coagulation and peripheral gangrene (25). The mean hemoglobin levels increased after subcurative antimalarial treatment but reached only 40 to 60% of the baseline hemoglobin levels. The animals received complete antimalarial treatment 25 days after the challenge infection (Fig. 1). The sharp drop in hemoglobin concentrations in association with relatively low parasitemia in all animals suggested that the major portion of the anemia was not due to erythrocyte destruction by parasitism but rather by hemolysis or an accelerated removal of uninfected erythrocytes.

Reticulocyte counts were also determined daily after experimental infection, as an indirect readout of the efficiency of erythropoiesis. Consistent with an adverse effect of malaria infection on erythroid progenitors, reticulocyte counts did not change from baseline levels. The proportion of reticulocytes in the peripheral blood increased only after rapid-acting antimalarial treatment was administered to a group of five malaria-naïve rhesus macaques, successfully study the erythrocyte life span, the biotin infusion was delivered intravenously using a Buretrol infusion set adjusted to deliver 40 to 50 microdrops/min. To prevent potential anaphylactic reactions, the animals were premedicated with diphenhydramine (10 mg intravenously [i.v.]). Clear distinctions between positive and negative cells were observed by flow cytometry using streptavidin-Alexa Fluor 488, with no significant differences observed between doses (data not shown). Based on these results, we decided to use the lower dose of 20 mg/kg in subsequent experiments. To determine if the procedure was adequately sensitive to successfully study the erythrocyte life span, the biotin infusion was administered to a group of five malaria-naïve rhesus macaques, and the proportions of biotinylated cells in the capillary blood were determined daily for 120 days (Fig. 2). The total number of biotinylated cells in the peripheral blood at different time points is presented for the individual animals with representative histograms. The kinetics of erythrocyte clearance determined from these experiments is in agreement with the reported erythrocyte life span in rhesus macaques, estimated to be ~100 days (30), with the percentage of Alexa Fluor-positive cells ranging between 1 and 3% of the total number of circulating cells in the peripheral blood at the end of the follow-up period.

Accelerated removal of uninfected erythrocytes in the course of experimental infection of rhesus macaques with P. coatneyi. Intravenous infusions of biotin were administered immediately before the experimental challenge of a new group of malaria-naïve rhesus monkeys by using the optimized dose of 20 mg/kg of EZ-Link Sulfo-NHS-biotin (noted above). The kinetics of parasitemia and the total number of biotinylated cells in the peripheral blood during the primary infection is summarized in Fig. 3. The para-
sitemias during these new infections were represented by slightly different kinetics from those described above. Maximum parasitemia occurred on day 10 with a mean of 38,000 parasites/μl. Biotinylated cells gradually decreased during the first few days following challenge infection, with a subsequent accelerated drop in the numbers of labeled cells between days 6 and 10. Biotinylated cells were not detected in the peripheral blood after 26 days postinfection. These results are consistent with an accelerated and complete turnover of all of the biotinylated cells (Fig. 3, right panel). This contrasts with the physiological clearance of 35% ± 5% of the erythrocytes in the uninfected control animals on day 26 (Fig. 3; \( P = 0.011 \)). The premature removal of the vast majority of biotinylated erythrocytes in the infected animals clearly occurred at the expense of uninfected erythrocytes and not through destruction from parasitism, given the relatively low level of parasitized cells during this portion of the acute infection.

**Partially immune rhesus macaques are resistant to severe anemia in *P. coatneyi* infections.** Clinical immunity to *P. falciparum* malaria is acquired after one or more homologous infections (31). To characterize the effect of clinical and antiparasite immunity on malaria severity, acquired by experimental exposure to *P. coatneyi*, the same group of rhesus macaques described above (Fig. 1) was reexposed to the same strain of parasite 9 months after the first infection in the previously naïve animals. The kinetics of parasitemia in the partially immune rhesus macaques followed a strikingly different pattern in comparison to the accelerated increase in parasite loads observed during the first infection. After being reinfected, the animals first exhibited parasitemia on days 5 and 7 postinfection with average levels of only 30 and 10 parasites/μl. The maximum peak of parasitemia was observed on day 14 with an average number of 40,000 parasites/μl (Fig. 4). Clinically, the outcome was also striking by comparison. The animals were assessed to be in fair condition and did not require antimalaria treatment or fluid support. Hemoglobin levels were maintained over 11 g/dl, with the lowest level recorded on day 17, when the animals reached 64% of the baseline hemoglobin levels with a mean value of 11.05 g/dl. The quantifying of reticulocytes in the peripheral blood showed an average peak of 17% reticulocytes on day 20, which corresponds to an average number of 735,678 reticulocytes/μl (Fig. 4, right panel). Interestingly, a progressive increase in the number of circulating reticulocytes coincided with a progressive decrease in hemoglobin levels. These results therefore suggested efficient compensatory mechanisms leading to effective erythropoiesis in the course of the second experimental infection.

The turnover of biotinylated erythrocytes was also quantified during the course of each subsequent second infection (conducted 9 months after the first infection) and compared with the data recorded from uninfected macaques (Fig. 5). In contrast to what was observed in the course of the first infections, a slow decrease in biotinylated cell counts was recorded, with removal of 60% ± 15% of the biotinylated cells by day 30 after infection. No clinical signs of severe infection were observed during the follow-up period (see below). Although the maximum peak of parasitemia was recorded on day 12 with 22,428 parasites/μl, a significant reduction in the number of biotinylated erythrocytes was observed in comparison to the unchallenged control group that received only...
a biotin infusion (Fig. 5; P = 0.01). Differences were also significant compared with the number of biotinylated cells recorded during the first infection (P = 0.0317). The lower level of hemoglobin was recorded 24 days after challenge with an average level of 9.7 g/dl. The animals recovered spontaneously without antimalarial chemotherapy or fluid support.

**Clinical assessment of malaria severity in rhesus macaques experimentally infected with *P. coatneyi***. Additional clinical and laboratory data were collected during the course of *P. coatneyi* infections in a new group of five malaria-naïve rhesus macaques, which also included daily monitoring of the erythrocyte life span by use of in vivo biotinylation procedures as described above. A control group of rhesus macaques were included to evaluate the normal variability in hematological and clinical laboratory parameters after biotinylation. To generate a daily quantitative assessment of the clinical severity and to facilitate comparison between groups, we also developed a rhesus physiological score (RPSS; Table 1). **Figure 6** summarizes the time course of the RPSS recorded during the first and second infections in comparison to clinical parameters recorded in the control group after in vivo biotinylation. In the course of the first experimental infection, the RPSS progressively increased to reach a mean peak value of 7.2 on day 10 (Fig. 6). The scores recorded during the second infection showed a significantly lower mean peak value of 4.6 on day 12. Differences in severity were noted between the first and second infections in these animals (Fig. 6; P = 0.016). As expected, the scores recorded in the control group showed similar patterns with no significant differences among them.

**Characterization of hemozoin deposition**. Epidemiological and experimental data in humans have shown that hemozoin pigment-containing leukocytes circulating in the peripheral blood are sensitive markers for parasite burden and disease severity, particularly when hemozoin-containing parasite stages are sequenced (32, 33). The proportion of hemozoin-containing monocytes and neutrophils was therefore determined daily in the course of the infections. Although the proportion of hemozoin-containing leukocytes increased with rising parasitemia, phagocytosis of hemozoin pigment by monocytes or neutrophils was positively correlated with parasitemia only in the course of the first infection (P = 0.007, r = 0.4818, and P < 0.0001, r = 0.717, respectively). Interestingly, drops in hemoglobin levels were negatively correlated with hemozoin-containing neutrophils (P = 0.0187, r = −0.426) but not with hemozoin-containing neutrophils. When the proportions of hemozoin-containing phagocytes were compared with the RPSS, statistically significant positive correlations were noted in the course of the first infection between phagocytes and the clinical scores (for monocytes, P < 0.0001, r = 0.7385; for neutrophils, P = 0.002, r = 0.5373). In the course of the second infection, a positive correlation was noted only between the frequency of hemozoin-containing monocytes and the clinical scores (P < 0.0001, r = 0.6720).

**Bone marrow abnormalities indicate dyserythropoiesis in the naïve rhesus macaques infected with *P. coatneyi***. Core bone marrow biopsy specimens were obtained at three different times during the course of the experimental infections to assess the possible impact of the *P. coatneyi* infections on bone marrow progenitors. The time points were selected based on the clinical response to the experimental infection: (i) baseline (BL); (ii) acute phase of the infection (clinical malaria [CM]), which coincides with the highest clinical score (RPSS); and (iii) 7 days after the highest RPSS was reached (after severe malaria [AM]). **Figure 7** includes images of representative core biopsy specimens.

Baseline bone marrow samples were normocellular with a 1:3 ratio of erythroid to myeloid series. During the acute phase of the infection, both the erythroid and myeloid series were expanded,
with a marked shift to immaturity consistent with erythroid and myeloid hyperplasia (Fig. 7B). Nevertheless, erythroid hyperplasia was associated with morphological changes consistent with dyserythropoiesis, including nuclear lobulation and nuclear fragmentation (karyorrhexis) (Fig. 7C). Seven days after antimalaria treatment, these nuclear changes were no longer observed, suggesting the return of effective erythropoiesis (Fig. 7D). Bone marrow biopsy specimens were also obtained in the course of the second experimental infection, using similar time points based on the RPSS scores. Histological analyses revealed erythroid hyperplasia with efficient erythroid differentiation (Fig. 7E and F).

**Severe malaria and multisystemic effect of *P. coatneyi* infections.** Peripheral blood samples for clinical chemistry, coagulation tests, and the characterization of proinflammatory mediators were collected at the same time points as described above for bone marrow core biopsy specimens and at the end of the follow-up period (EF). Clinical laboratory tests confirmed progression to severe disease in the course of the first infection with azotemia, elevated levels of creatinine phosphokinase (CPK), hypertriglyceridemia, and hypoalbuminemia, which were significantly different in comparison to baseline levels (Fig. 8). Differences in blood urea nitrogen (BUN) and CPK levels were also significantly different in comparison to the levels in the same monkeys during the course of the second infection. Interestingly, hypoalbuminemia was also observed during the second infection, with significant differences compared to baseline levels (Fig. 8). Although differences in the triglyceride levels during the second infection were not significant in comparison to baseline levels, there was a trend toward hypertriglyceridemia (Fig. 8).

We have reported that thrombocytopenia and severe coagulopathy can be induced in rhesus macaques experimentally infected with *P. coatneyi* (25). To determine whether the activation of the coagulation cascade is a common finding in this experimental model, coagulation profiles were assessed in the course of the experimental infections using fresh plasma samples to record prothrombin time (PT), partial-thromboplastin time (PTT), fibrinogen, fibrin degradation products, and inhibitors of coagulation. No significant changes were observed for PT and PTT. As expected for an acute-phase reactant, plasma fibrinogen levels were elevated in infected macaques, and these changes coincided with increases in the number of parasites in the peripheral blood (data not shown). Significant increases in the levels of D-dimers in com-
The clinical and laboratory tests described above suggested progression to severe disease in the course of the infections in the malaria-naïve individuals, and each of these animals required subcurative antimalarial treatment to avoid life-threatening complications. Given the clinical prognosis, four of the five animals required intravenous fluid support and also received whole-blood transfusion with the recommendations and oversight from the veterinarian support staff. This clinical outcome is in sharp contrast with the clinical evolution of the subsequent experimental infections of these animals. Like in the first group of rhesus macaques after a subsequent second infection was given, these animals also did not require antimalarial chemotherapy or fluid support and they recovered naturally.

**Proinflammatory response in *P. coatneyi*-infected rhesus macaques.** Severe malaria in humans is associated with a strong proinflammatory immune response. To evaluate the magnitude of the proinflammatory response in the rhesus macaques experimentally infected with *P. coatneyi*, using multiplex assays, we determined the levels of 18 factors, including cytokines, chemokines, and metalloproteinases in serum samples from four time points. Eight factors were significantly different compared to baseline levels (Fig. 10). We identified increased levels of IFN-γ, IL-6, TNF-α, MIP-1β/CCL4, MCP-1/CCL2, TIMP1, and NAP2. These proinflammatory mediators were upregulated when severe malaria episodes were recorded using the RPSS during the course of the first infection (Fig. 10A).

The mean concentration of IFN-γ in serum was 105 pg/ml in the first infection and 67 pg/ml in the second infection, which is significantly different for the first infection compared to baseline values (*P* < 0.0001). The mean level of IL-6 was 19.4 pg/ml in the first infection and 1.5 pg/ml in the second infection, which was significantly different for the first infection compared to baseline values (*P* < 0.01). The mean level of TNF-α in serum was 175 pg/ml in the first infection and 39 pg/ml in the second infection, with a significant difference for the first infection compared to baseline values (*P* < 0.001). The mean concentration of MIP-1β/CCL4 was 547 pg/ml during the first infection and 90 pg/ml in the second infection, which was a significant difference in the first infection compared to baseline values (*P* < 0.001). The mean level of MCP-1/CCL2 in serum was 1,607 pg/ml in the first infection and 65 pg/ml in the second infection, with significant differences for the first infection compared to baseline values (*P* < 0.01). The mean level of TIMP-1 was 2.27 µg/ml in the first infection and 0.4 µg/ml in the second infection, with a significant difference for the first infection compared to baseline value (*P* < 0.0001). The mean fold increase of NAP-2 during the first infections compared to baseline levels was 8 (*P* < 0.05) (Fig. 10A). Levels of IL-6, TNF-α, MIP-1β, and MCP-1 in serum decreased 7 days after provision of antimalarial treatment with artemether in the first infection (Fig. 10B). Mean fold increases of IFN-γ, TIMP-1, and NAP-2 were significant in the first infection in comparison to baseline levels at this posttreatment time point (*P* < 0.05, *P* < 0.01, and *P* < 0.0001, respectively.). Interestingly, levels of MMP-9 in serum increased by the end of the follow-up of the first infection to reach a mean fold increase of 16 with respect to baseline levels (Fig. 10B; *P* < 0.01).

**Erythropoietin (EPO) levels in *P. coatneyi*-infected rhesus macaques.** To determine if the indications of dyserythropoiesis seen in the bone marrows from *P. coatneyi*-infected rhesus mon-
 keys are due to a deficiency in EPO production, we quantified the levels of EPO in serum samples using a commercially available cross-reactive human immunoassay. The serum samples tested were obtained at the same time points as described above for bone marrow core biopsy specimens. Comparable baseline EPO levels were recorded before biotin infusion with values ranging between 13.5 and 40.2 mUI/ml. However, EPO levels were 6-fold higher in the \textit{P. coatneyi}-infected rhesus macaques than in the naïve group at the time of the maximum drop in hemoglobin levels. EPO levels in serum in the first infections at this time ranged between 207 and 307 mUI/ml, in contrast to the levels that ranged between 9 and 80 mUI/ml during the second infection. EPO levels decreased sharply 7 days after the single artemether treatment was given, with values ranging between 39 and 120 mUI/ml for the first infection. This is in contrast with the 10- to 17-fold increases above baseline EPO levels in serum in the first infections at this time.

FIG 9 Increased coagulation activity in \textit{P. coatneyi}-infected rhesus macaques. Time course levels of D-dimers, protein C, and protein S in rhesus macaques exposed to the experimental infections. Data are from individual animals and group means (dotted bars) at the following time points: at baseline (BL), before parasite inoculation; at the time when RPSS clinical malaria (CM) was recorded; 7 days after the episode of severe malaria (AM); and at the end of the follow-up period (EF). Closed symbols, first infection; open symbols, second infection. ANOVA with Bonferroni’s multiple-comparison posttest was used to compare changes after log transformation. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\).

FIG 10 \textit{P. coatneyi} induces strong inflammatory immune response in malaria-naïve rhesus macaques. Individual patterns of plasma inflammatory mediators are compared in the course of the first (closed symbols) or second (open symbols) infections. (A) Samples obtained when high score values were detected using RPSS. (B) Samples obtained 7 days after the RPSS peaked. The results are expressed as fold increases of the corresponding mediator over baseline levels. Horizontal bars represent arithmetic mean values for each group. ANOVA with Bonferroni’s multiple-comparison posttest was used to compare changes in baseline levels after log transformation. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\).

FIG 11 Ineffective erythropoiesis in \textit{P. coatneyi} rhesus macaques is not dependent on abnormal EPO production. Time course levels of erythropoietin in plasma samples obtained from rhesus macaques in the course of the first infection (closed symbols) or second infection (open symbols) determined by immunoassay. Data present levels for individual animals at baseline (BL), before parasite inoculation; at the time when RPSS clinical malaria (CM) was recorded; 7 days after the episode of severe malaria (AM); and at the end of the follow-up period (EF). Horizontal bars represent arithmetic mean values for each group. ANOVA with post hoc Bonferroni’s multiple-comparison posttest was used to compare changes after log transformation. **, \(P < 0.01\); ***, \(P < 0.001\).

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hemoglobin levels in the course of the first infection but not in the second infection (\(r = -0.902, P < 0.0001\)). These results suggest that the homeostatic mechanism of production of EPO in response to the acute anemia stress is operational and that the absence of EPO was not a factor in the dyserythropoiesis described in this model.

DISCUSSION
Severe malaria is the result of extensive and excessive metabolic dysfunction that leads to a variety of clinically heterogeneous syn-
dromes. Although adhesion-mediated compartmentalization of infected erythrocytes in microvascular beds of the brain and placental milieu supports a mechanistic role for sequestration in the pathophysiology of *P. falciparum* cerebral and placental malaria (34–37), the complexity of the clinical presentation of severe malaria clearly indicates that additional factors beyond vascular adhesion are involved (38). In fact, for both *P. falciparum* in humans or *P. coatneyi* in rhesus monkeys, profound sequestration of infected erythrocytes occurs in many tissues in uncomplicated malaria infections. It is not known for certain that enhanced sequestration of infected erythrocytes in particular compartments such as brain or lung directly leads to cerebral malaria or acute respiratory distress, respectively, or if other pathogenic factors that lead to the observed sequestration in these tissue compartments in severe malaria are activated (39). Severe malaria cases reported for the nonsequestering human malaria species, in particular *P. vivax* and *P. knowlesi* (40–42), also suggest the role of unknown mechanisms and mediators that are difficult to discern based solely on data derived from clinical cases.

Experimental animal models have the potential to identify factors involved in initiating and accelerating the pathogenesis of severe malaria, which will be important for developing novel interventions and adjunctive therapies (8–10). The most characterized experimental animal model of severe malaria is the rodent *P. berghei* ANKA mouse model that was proposed for human cerebral malaria (43, 44). However, the ANKA model has unique histological findings, such as the accumulation of leukocytes, that have not been reported in human cerebral malaria, which is characterized by the adhesion of infected erythrocytes to the endothelium of brain vessels (8). The model is therefore appropriate to test interventions aimed to reduce local inflammatory reaction (8), but this model may not adequately mimic the pathological pathways responsible for the syndromes that are invoked in human severe or even cerebral malaria and has been called into question (7). This is in contrast with the evidence of infected erythrocyte sequestration and rosetting reported in nonhuman primate models infected with *P. fragile* and *P. coatneyi* that mimic the patterns of tissue sequestration seen in humans infected with *P. falciparum* (14, 17, 45–47). These findings provide a level of support for the use of nonhuman primate models to study the complexity of the host-parasite interaction.

Here we add further support by demonstrating that malaria-naïve rhesus macaques experimentally infected with *P. coatneyi*-infected erythrocytes develop severe anemia, coagulopathy, renal impairment, and general metabolic dysfunction, outcomes frequently encountered in severe malaria in humans (48). During the course of the acute *P. coatneyi* infections, clinical complications developed and required antimalaria chemotherapy, fluid support, and whole-blood transfusion, mimicking the clinical care provided to treat severe malaria in humans (5). Signs of severe malaria were observed with relatively low parasitemia, resembling situations observed in nonimmune children in areas of unstable malaria transmission (49). Subsequent infections showed that acquired immunity partially protects against severe malaria.

To investigate the mechanistic basis of severe anemia using the *P. coatneyi*-rhesus monkey model, we studied the erythrocyte life span and bone marrow core biopsy specimens. The experimental infection of malaria-naïve rhesus macaques resulted in the pronounced accelerated reduction in hemoglobin levels that led to severe anemia 10 to 12 days after experimental infection. The accelerated turnover of erythrocytes occurred at relatively low parasite burdens and could not be accounted for by the cumulative destruction from parasitism, which suggested that the majority of erythrocyte clearance happened at the expense of uninfected erythrocytes. Our data showed that in the course of the first infection the life span of uninfected erythrocytes was shortened by about 80%. To our knowledge, this is the first time that removal of uninfected erythrocytes in the course of malaria infection has been experimentally determined in nonhuman primates.

The massive destruction of uninfected erythrocytes greatly increased EPO production with the subsequent hyperplasia of erythroid progenitors. However, distinct dyserythropoietic changes that coincided with an observed reticulocytopenia with relatively low parasitemias were noted. These results demonstrate that erythropoiesis is severely diminished in primary infections of *P. coatneyi* in rhesus macaques. Our results contrast with those reported in the *P. chabaudi* AS mouse model, in which suppression of erythropoiesis has been associated to hyperparasitemia (50, 51). Our data are also in contrast to those reported in the *P. berghei* ANKA semi-immune mouse model, in which destruction of uninfected erythrocytes appears to be solely responsible for the severe anemia (28, 52), perhaps because the spleen and, to a lesser extent, the liver are major hemopoietic tissues in mice but not primates. Suppression of erythropoiesis with bone marrow hyperplasia accompanied by dyserythropoiesis and inefficient reticuloeyte production index has been described in patients with severe malarial anemia (53–55). Acquired immunity in *P. coatneyi*-infected rhesus monkeys is not sterilizing and can be used to mimic chronic infections that reproduce what is seen in children in areas of high stable transmission (21). Taken together, the data support the use of *P. coatneyi* in rhesus macaques to study the molecular mechanisms involved in severe malarial anemia.

Our data on the reduced erythrocyte life span in *P. coatneyi*-infected rhesus macaques conform with epidemiological data suggesting that the destruction of uninfected erythrocytes accounts for more than 90% of the erythrocytes lost in malaria infections (56). Although reduced erythrocyte life span was also recorded in our study in the course of the second infection, the reduction was less severe; an average of only 10% of biotinylated cells were detected on day 68 after infection, indicating that the removal of uninfected erythrocytes was still occurring even after completion of antimalaria chemotherapy. Data derived from naturally infected individuals also indicate that after parasite clearance the erythrocyte life span is reduced in patients recovering from *P. falciparum* or *P. vivax* malaria (57, 58). Hypotheses put forth for the destruction of uninfected erythrocytes have focused on different possible mechanisms including reduced erythrocyte deformability (59, 60), accelerated erythrocyte senescence (61), and immunologic removal (62–66), although none have been conclusively proven to be the mechanism.

In Gabonese children with *P. falciparum* malaria, proinflammatory responses have been shown to activate macrophages to release oxygen and nitrogen radicals that can cause oxidative damage of infected and uninfected erythrocytes (67). Erythrocyte clearance in these situations involves the removal of both infected and uninfected cells by erythropagocytosis mediated by macrophages. Erythropagocytosis is an effector mechanism that can be modulated via the engagement of several receptors expressed on mononuclear phagocytes. Antibody-erythrocyte antibodies can engage CD16/FcγRIIA on monocytes, leading to anemia. Interestingly,
studies in Kenya have confirmed that children at risk of developing severe malarial anemia overexpressed CD16/FcγRIIa in response to the infection, in contrast to children who develop other forms of clinical malaria (68). Erythropagocytosis can also be modulated by activation of the complement cascade. This homeostatic process is down-modulated by regulatory proteins that include complement receptor-1 (CR1), decay-accelerating factor (DAF or CD55), and membrane inhibitor of reactive lysis (MIIRL or CD59) (reviewed in reference 69). It has been demonstrated that the levels of such regulatory proteins are inversely correlated with malaria severity, suggesting that erythrocytes from nonimmune individuals could be more susceptible to complement-mediated lysis than erythrocytes of immune individuals (62). However, intravascular erythrocyte lysis aside from that caused by parasitism is not evident in severe anemia in humans or monkey infections, and Coombs’ positive reactions in malaria infections do not correlate with the excessive loss of erythrocytes, which suggests the participation of different mechanisms in erythrocyte clearance (70). Interestingly, biochemical modifications that mimic the physiological aging process are induced in the membrane of infected and uninfected erythrocytes in vitro (71), and similar changes have been reported in vivo in P. knowlesi-infected rhesus macaques (72). The definition of the mechanistic basis involved in erythrocyte clearance in severe malaria anemia requires further investigation.

Erythropoietin (EPO) is an essential growth factor that promotes erythropoiesis by supporting proliferation, differentiation, and maturation of erythroid progenitors. Results in humans have been reported indicating that levels of EPO are elevated in children with uncomplicated P. falciparum malaria (73). However, levels of EPO seem to be reduced relative to the severity of anemia in adults with malarial anemia (74, 75). We have shown here that EPO levels were associated with malaria severity in rhesus macaques experimentally infected with P. coatneyi. The inverse correlation between hemoglobin and EPO in the course of the first infection but not during the second suggests suboptimal erythropoietic responses in the naïve animals. Differences in the severity of malarial anemia between malaria-naïve and partially immune rhesus macaques can therefore be explained by erythropoietic suppression. Our results are in agreement with recent data from an area of endemicity with perennial transmission indicating that EPO levels increased according to malaria severity (76).

The production of several immune mediators is essential for parasite clearance. However, proinflammatory mediators are also involved in malaria pathogenesis. We investigated the production of 18 proinflammatory mediators. Significant differences in the plasma levels of eight proinflammatory mediators tested were detected in rhesus macaques in the course of the first P. coatneyi infection in comparison to baseline levels (IFN-γ, IL-6, TNF-α, MIP-1β/CCL4, MCP-1/CCL2, TIMP1, NAP-2, and MMP-9). These changes were evident only at the time when clinical severity scores reached a peak. With the exception of IFN-γ, TIMP-1, and NAP-2, no significant differences from baseline values were found in samples obtained 7 days after reaching the maximum clinical scores. IFN-γ is essential for protection in clinical malaria (77,78); in fact, early production of IFN-γ is a critical factor in the differences in susceptibility to malaria reported among sympatric ethnic groups living in Mali (79, 80). However, overproduction of IFN-γ has also been associated with anemia, exemplified by bone marrow suppression, dyserythropoiesis, and erythropagocytosis (81). Similarly, high levels of TNF-α, associated with different inflammatory entities, inhibit erythropoiesis (82), having an impact on cell cycle progression (83), modulating transcription factors essential for erythroid differentiation (84), and promoting erythropagocytosis (85). TNF-α is also associated with dysfunction of multiple organs, leading to cerebral malaria, respiratory distress, hypoglycemia, and placental pathology (86, 87). High levels of IL-6 have been reported in patients with severe malaria compared to the matched uncomplicated malaria cases (88, 89). Along with TNF-α and IL-1, IL-6 is a pyrogen and inducer of the acute-phase response (90). A recent prospective cohort study showed that IL-6 is a strong predictor of malaria clinical infections (91). Interestingly, genetic population studies in East Africa have indicated a high-frequency distribution of genotypes associated with low expression of IL-6 in children over 12 years old and young adults, indicating a positive selection early in life (92).

Among the chemokines analyzed, high levels of MCP-1/CCL2, MIP-1β/CCL4, and NAP-2 were recorded in P. coatneyi-infected rhesus macaques. High levels of MCP-1/CCL2 and MIP-1β/CCL4 have been reported to be associated with hyperparasitemia and placentation pathology (93–95). Children with severe P. falciparum infections associated with cerebral malaria and mortality had higher serum levels of MIP-1β/CCL4 than did children who survived (96). Consistent with the role of MIP-1β/CCL4 in cerebral malaria pathogenesis, high levels were reported in postmortem cerebrospinal fluid samples obtained from children with cerebral malaria compared with samples obtained from children that died of severe malarial anemia (97).

Interestingly, to our knowledge this is the first time that the chemokine CXCL7, the most abundant platelet chemokine, present in the α-granules (98). NAP-2 induces neutrophil adhesion to endothelial cells and transendothelial migration and has been involved in regeneration of vascular integrity after injury. Studies with malaria-naïve volunteers experimentally infected with P. falciparum have shown that levels of CXCL7 are elevated in the early stages of the infection (99). Given the evidence of thrombocytopenia and coagulopathy described here for P. coatneyi infections, and as we have reported for a severe case of disseminated intravascular coagulation (DIC) with P. coatneyi (25), high levels of NAP-2 in plasma could indicate massive platelet activation. In fact, high levels of platelet membrane products in plasma have been used as evidence of in vivo platelet activation (100).

Matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of matrix metalloproteinases [TIMPs]) have been reported to play a role in the inflammatory response (reviewed in reference 101). MMPs are essential for leukocyte traffic, modulation of proinflammatory mediators, and tissue repair. High levels of TIMP-1 and MMP-8 have been reported in serum samples from patients with severe or uncomplicated P. falciparum malaria (102). Here we demonstrate that malaria severity in the course of the first P. coatneyi infection was associated with high levels of TIMP-1. High levels of TIMP-1 in plasma were also recorded at two subsequent time points after subcurative treatment. Plasma levels of MMP-9 were elevated in the first infection at the end of the follow-up period. These results could indicate that in our NHP model TIMP-1 is involved in malaria pathogenesis, whereas MMP-9 could be involved in tissue regeneration (103). Interestingly, MMP-9 has a platelet antiaggregation effect and plays a role
in thrombopoiesis (104). It is therefore feasible that increasing levels of MMP-9 after several days of subcurative antimalarial chemotherapy were in response to thrombocytopenia and massive endothelial cell activation. MMP-9 has been proposed as a biomarker of severe malaria and is a good candidate for targeted therapy (105). Our data suggest that MMP-9 is involved in a counterregulatory activity of proinflammatory mediators. Such results coincide with the reported failure to demonstrate a correlation of MMP-9 sera or cerebrospinal fluid levels in patients with severe malaria (102, 106). The clinical outcome of malaria infections depends on the appropriate balance between proinflammatory and counterregulatory mediators. Understanding their role in clinical disease could provide important insights for the development of adjunctive chemotherapy.

Hemozoin, an insoluble by-product of hemoglobin proteolysis, has been reported to exhibit several biological effects that involve induction of proinflammatory mediators (reviewed in reference 107), dysfunction of monocyte/macrophages (108), modulation of the maturation of dendritic cells (109, 110), immunosuppression (111, 112), and suppression of erythropoiesis (113–117). To investigate the role of hemozoin deposition in the pathogenesis of severe malaria, we characterized the relationship between pigment-containing leukocytes, hemoglobin levels, and clinical severity. The proportion of hemozoin-containing phagocytes correlated with clinical severity and parasitemia in the course of the first infection. This is consistent with reports of high mortality in patients with severe malaria associated with a high proportion of hemozoin-containing neutrophils and monocytes in comparison to patients with severe malaria that survive (32). Similar findings have also been reported in children (118, 119). However, most recently, in a comprehensive study involving six sites in five different countries, broad heterogeneity was shown among the sites, suggesting that circulating hemozoin-containing phagocytes have limited value as a predictor of fatal outcome (33). Abnormal erythroid progenitors have been associated with hemozoin-containing myeloid cells in postmortem bone marrow biopsy specimens derived from children with severe malarial anemia (113). The inhibitory role of hemozoin on erythropoiesis has also been characterized using in vitro culture systems showing a direct effect through inhibition of erythroid cell development (114, 117), growth inhibition by down-modulation of the expression of several receptors critical for differentiation (117), induction of apoptosis (116), and indirectly through the activity of inflammatory mediators (114). To determine if suppression of erythropoiesis in malarial anemia can be associated with hemozoin deposits in bone marrow, we evaluated the accumulation of hemozoin in the rhesus bone marrow samples using cell image analysis. Similar patterns of hemozoin accumulation were determined in bone marrow biopsy specimens derived from both the first and the second experimental infections (data not shown). Our data suggest that hemozoin deposits were not responsible for the observed effect on erythroid progenitors. The robust erythropoietic response after antimalaria chemotherapy also suggests that unidentified bioactive molecule(s) could play a role in dyserythropoiesis during severe malaria. The NHP model will be useful in future studies aiming to identify potential parasite bioactive products using high-throughput technologies.

The mean triglyceride levels in infected animals were significantly higher than baseline levels in the course of the first P. coatneyi infections. In retrospective studies, hypertriglycerideremia has been described in patients infected with P. falciparum (120, 121). Interestingly, high levels of triglycerides were also reported to be associated with severe malaria (122, 123). In these studies, significant differences in the levels of triglycerides were defined in travelers returned from areas of endemicity with severe malaria in comparison to returned travelers with nonsevere malaria. In our experimental model, differences in mean triglyceride levels were not significant when first and second infections were compared, suggesting that malaria severity was not associated with hypertriglycerideremia in rhesus macaques infected with P. coatneyi (Fig. 8). The mechanism underlying changes in triglycerides involves increases in the production of very-low-density lipoprotein (VLDL) as a result of mobilization of free fatty acids from adipose tissue in response to stress (120). Metabolic alterations mediated by malaria parasites such as the reduction in lipoprotein lipase (LPL) activity may also account for the reduction in triglyceride clearance (124). Interestingly, TNF-α has been associated with LPL suppression (125). Here we have shown that the first experimental infection with P. coatneyi resulted in a 9- to 61-fold increase in TNF-α levels compared to baseline levels, whereas the second infection resulted in a 2- to 21-fold increase in four of the five animals tested. In vitro evidence indicates that hypertriglyceridemia modifies the pharmacokinetics of lipophilic antimalarial drugs (126). Hypertriglycerideremia in the course of malaria infection might therefore have an impact on chemotherapy efficacy.

Hypoalbuminemia was identified in the course of the first and second P. coatneyi infections of the rhesus macaques. In contrast to triglyceride levels, albumin levels were also significantly lower than baseline levels at the end of the follow-up period, confirming that the inflammatory disorder was more severe in the course of the first infection. Severe malaria is associated with changes in capillary permeability that mimic what has been described in severely ill patients with sepsis (127). Increased vascular permeability has been previously demonstrated in rhesus macaques experimentally infected with P. coatneyi (128). In addition, the decrease of the hepatic synthesis of albumin due to increased synthesis of acute-phase proteins may also play a role in decreased albumin concentration in severe malaria (129). Hemodynamic changes that resulted from hypoalbuminemia and vascular dysfunction are critical factors for the development of pulmonary edema (130).

In conclusion, we have presented comprehensive clinical and laboratory parameters from rhesus macaques experimentally infected with P. coatneyi. This study represents the first in-depth assessment and evidence showing that this simian malaria parasite can induce severe anemia, coagulopathy, renal impairment, and a generalized metabolic dysfunction comparable to what is seen in human cases. Our data support the use of this parasite-host combination to study the molecular mechanisms involved in the pathogenesis of severe malaria. Finally, it is worth noting that other simian Plasmodium species such as P. cynomolgi, a parasite biologically and phylogenetically closer to P. vivax (131), offers a unique opportunity for future comparative and coinfection studies to better understand the complex parasite-host pathophysiological interactions that operate in human and nonhuman primate malaria infections.

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Infection and Immunity


Consider the following scenario:

John, a pediatrician, is examining a patient with symptoms suggestive of Plasmodium falciparum infection. The patient is suspected to have malaria, characterized by fever, anemia, and altered mental status. John decides to perform a serological assay to detect antibodies specific to Plasmodium falciparum. He collects a blood sample from the patient and sends it to the laboratory for testing. The laboratory processes the sample and performs the assay, which is designed to detect antibodies against Plasmodium falciparum. Upon receiving the results, John interprets the findings and determines the presence or absence of specific antibodies associated with malaria. Based on the laboratory report, John makes a diagnosis and initiates appropriate treatment for the patient. This scenario highlights the importance of serological assays in the diagnosis and management of malaria, particularly in regions where the disease is endemic. John's approach underscores the role of laboratory investigations in confirming the clinical diagnosis and guiding the therapeutic strategy.


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