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Both Hemolytic Anemia and Malaria Parasite-Specific Factors Increase Susceptibility to Nontyphoidal *Salmonella enterica* Serovar Typhimurium Infection in Mice[▽]

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Severe pediatric malaria is an important risk factor for developing disseminated infections with nontyphoidal *Salmonella* serotypes (NTS). While recent animal studies on this subject are lacking, early work suggests that an increased risk for developing systemic NTS infection during malaria is caused by hemolytic anemia, which leads to reduced macrophage microbicidal activity. Here we established a model for oral *Salmonella enterica* serotype Typhimurium challenge in mice infected with *Plasmodium yoelii nigeriensis*. Initial characterization of this model showed that 5 days after coinoculation, *P. yoelii nigeriensis* infection increased the recovery of *S.* Typhimurium from liver and spleen by approximately 1,000-fold. The increased bacterial burden could be only partially recapitulated by antibody-mediated hemolysis, which increased the recovery of *S.* Typhimurium from liver and spleen by 10-fold. These data suggested that both hemolysis and *P. yoelii nigeriensis*-specific factors contributed to the increased susceptibility to *S.* Typhimurium. The mechanism by which hemolysis impaired resistance to *S.* Typhimurium was further investigated. *In vitro*, *S.* Typhimurium was recovered 24 h after infection of hemophagocytic macrophages in 2-fold-higher numbers than after infection of mock-treated macrophages, making it unlikely that reduced macrophage microbicidal activity was solely responsible for hemolysis-induced immunosuppression during malaria. Infection with *P. yoelii nigeriensis*, but not antibody-mediated hemolysis, reduced serum levels of interleukin-12p70 (IL-12p70) in response to *S.* Typhimurium challenge. Collectively, studies establishing a mouse model for this coinfection suggest that multiple distinct malaria-induced immune defects contribute to increased susceptibility to *S.* Typhimurium.

The most frequent presentation of disease from nontyphoidal *Salmonella* serotypes (NTS) in developed countries such as the United States is gastroenteritis, a localized infection with low mortality. In contrast, immunocompromised individuals are at risk of developing NTS bacteremia, which can lead to serious sequelae, including meningitis, osteomyelitis, and septic shock (46). While these complications are infrequent in developed countries, NTS serotypes have become a leading cause of bacteremia in sub-Saharan Africa (12). The most frequent NTS isolates in this region are *Salmonella enterica* serotype Typhimurium and *S. enterica* serotype Enteritidis (4, 18). NTS bacteremia is most common in children younger than 5 years of age, with a peak prevalence for children 10 to 14 months of age (14). The main risk factors for NTS bacteremia are malnutrition, human immunodeficiency virus (HIV) infection, and malaria (5). The high prevalence of multidrug-resistant NTS in this region has made the treatment of NTS bacteremia even more difficult (13, 18, 39).

A factor that may contribute to the development of NTS bacteremia in pediatric malaria patients is hemolytic anemia. Studies from tropical Africa point toward a strong association of NTS bacteremia with severe malarial anemia (14). NTS bacteremia in both adults and children is also associated with other conditions resulting in anemia, such as sickle cell disease (36, 37). Hemolytic anemia is also a hallmark of pediatric malaria and occurs in part due to the clearance of damaged or parasitized erythrocytes from the circulation by the spleen (8, 38, 58). Previous experimental studies by Kaye and colleagues have shown that antibody-induced hemolysis prior to infection of mice with *S.* Typhimurium leads to a more rapidly fatal disease (19, 20). In contrast, blood loss due to hemorrhage did not lead to significantly higher rates of mortality during *S.* Typhimurium infection. These studies suggested that hemolysis may lead to a decreased bactericidal function of macrophages, rendering them unable to kill *Salmonella*.

The goal of our study was to investigate the pathogenesis of *S.* Typhimurium/malaria coinfection in a mouse model to explore the possible mechanisms that may cause an increased susceptibility to NTS septicemia in pediatric malaria patients.

MATERIALS AND METHODS

Bacterial and parasite strains. *S.* Typhimurium strain IR715 is a fully virulent, nalidixic acid-resistant derivative of strain ATCC 14028 (55). The rodent malaria parasite *Plasmodium yoelii nigeriensis* was obtained from the Malaria Research and Reference Reagent Resource (MR4) Center.

Molecular confirmation of *P. yoelii nigeriensis* infection. The frozen blood stock obtained from MR4 was derived from an isolate from the thicket rat, *Thamnomys rutilans*, in the early 1970s, prior to the availability of molecular biological tools for genetic characterization. In addition, cross-contamination of multiple lines of murine parasite species has confounded numerous studies (44, 47, 51). Therefore, to confirm the identity of our parasite stock, we designed 10 PCR primers against sequence regions of the gene encoding merozoite surface

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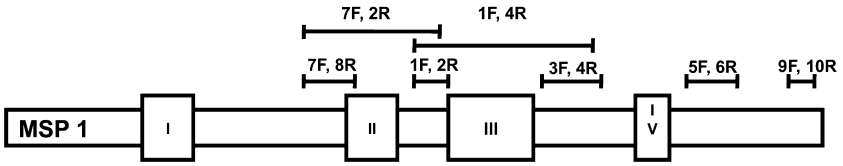

FIG. 1. Strategy for determination of species of murine malaria parasites. Boxes I, II, III, and IV represent variable regions of merozoite surface protein 1 (MSP-1), while the flanking regions are conserved among the different murine *Plasmodium* species (7). Brackets indicate the primer pairs (primers 1 to 10) used to verify species and subspecies.

protein 1 (*MSP-1*) that are highly conserved among the rodent parasite species *Plasmodium berghei* (GenBank accession number U43521.1), *Plasmodium chabaudi* (accession number L22982.1), and *Plasmodium yoelii* (accession number XM721164.1). The forward (F) and reverse (R) primer sequences were as follows: 5′-CAAAACTCTACCATAAACAAAGATGC-3′ (1F), 5′-TTTGTAA ACCATCAACTACATTTTCA-3′ (2R), 5′-AATGCATTGACCCCTGAAA A-3′ (3F), 5′-TCGGCTGTATGCTCTGAATTT-3′ (4R), 5′-TGATTTCCTTG ATGTATTAAGCTATGA-3′ (5F), 5′-AACTGCGTTTAATTGAGGTTTG-3′ (6R), 5′-GCAAATGCTGTTCAAGCAAA-3′ (7F), 5′-TGGCAAGTCTGTCA ATTTCTTT-3′ (8R), 5′-ATGCTTACCATGGATGGTATGGAT-3′ (9F), and 5′-ATAATCCCATAAAGCTGGAAGAACT-3′ (10R). These primers were used in multiple combinations to amplify conserved and variable regions of the *MSP-1* sequence that have been used to distinguish among rodent malaria parasite species, subspecies, and strains (2, 7, 9) (Fig. 1). At least 5 amplimers from each set of primers used with template DNA prepared from frozen stock, from passaged fresh blood, and from infected mouse tissues were submitted for direct sequencing to determine the clonality and identity of the parasite stock. Sequence analyses of our *MSP-1* amplimers confirmed that our parasite isolate was a clonal isolate of *P. yoelii nigeriensis*. All primer combinations yielded products, verified by gel electrophoresis, except for primers 5F and 6R, likely due to key nucleotide differences among species. Among the sequenced replicate amplimers for each primer pair, we detected only single sequences, suggesting that our stock was a clonal isolate. Based on BLAST analysis (1) and visual inspection of aligned sequences, our parasite isolate matched most closely with *Plasmodium yoelii* (Fig. 1). To confirm the parasite subspecies, we designed additional primers based on the variation among parasite strains in the sequence encoding the carboxy-terminal region of MSP-1, as described previously (9). The sequence of the 9F-10R amplimer was 100% identical to the reported sequence for *P. yoelii nigeriensis* (GenBank accession number M87557) and shared 96% identity with *P. yoelii yoelii* 17XNL (accession number XM_721164), 83.3% identity with *P. chabaudi chabaudi* (accession number L22982), and 89.4% identity with *P. berghei* ANKA (accession number U43521).

Animal experiments. Four-week-old female CD1 mice were provided by the UC Davis Center for Laboratory Animal Science. They were infected by intraperitoneal (i.p.) injection with a thawed aliquot of *P. yoelii nigeriensis*. Blood was collected when parasitemia was 10 to 15%, as determined by thin blood film analysis. Female CBA/J mice from the Jackson Laboratories (Bar Harbor, ME) were used at the age of 6 to 8 weeks. They were injected i.p. with 5×10^6 parasitized red blood cells (RBC) per mouse or an equivalent volume and preparation of uninfected blood. Mice were subsequently inoculated intragastrically (i.g.) by a feeding needle with 1×10^7 to 1×10^8 CFU of *S*. Typhimurium per mouse. To induce hemolysis, CBA/J mice received two i.p. injections of 150 μg of the rabbit IgG fraction of anti-mouse RBC (Rockland, Inc., Gilbertsville, PA) 2 days apart. As a control, mice received nonimmune rabbit IgG (Innovative Research, Southfield, MI). Two hours after the first injection, mice were inoculated with 1×10^8 CFU of *S*. Typhimurium per mouse. In all animal experiments, mice were sacrificed 5 days after *S*. Typhimurium infection. Spleen, liver, and Peyer's patches (PP) were collected aseptically in phosphate-buffered saline (PBS), homogenized, diluted, and plated onto Luria-Bertani (LB) agar supplemented with nalidixic acid. After overnight incubation at 37°C, CFU were enumerated. For analysis of changes in gene expression after infection, a portion of the spleen was snap-frozen in liquid nitrogen and stored at −80°C.

Histopathology. Samples of the spleen were fixed in buffered formalin, processed according to standard procedures for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin (HE). A veterinary pathologist (T.A.P.) examined the slides using a blinded-sample analysis.

Hematological analysis. Blood samples were collected from the saphenous vein into heparinized tubes. Blood was diluted in PBS, and erythrocytes were counted by using a hemocytometer.

Monitoring of *P. yoelii nigeriensis* parasitemia. On a daily basis, thin film blood smears were prepared and read to determine the percentage of parasitized red blood cells. A total of 3,000 cells in 5 fields were counted per animal per time point.

Erythrophagocytosis by J774.A1 macrophages. Sheep RBC were centrifuged at $1,000 \times g$ for 10 min at 4°C. An equal volume of Alsever solution (Sigma) was added after the removal of the plasma and buffy coat. The wash was repeated twice. For opsonization, 10^9 RBC were incubated with the rabbit anti-sheep IgG fraction (1:64 in PBS) (Rockland, Inc., Gilbertsville, PA) for 15 min at 37°C and then washed twice in 20 volumes of Alsever solution and resuspended in culture medium. J774.A1 macrophages were seeded in 96-well plates at a concentration of 7×10^4 macrophages/well in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, and 1 mM glutamine and incubated with RBC (multiplicity of infection [MOI] of 20) for 2 h at 37°C. Noningested RBC were removed with a 10-s hypotonic wash with sterile H_2O, followed by a wash with PBS. Macrophages were then infected with *S*. Typhimurium (MOI of 5), centrifuged for 5 min at 1,000 rpm, and incubated for 30 min at 37°C. Cells were washed once with PBS and incubated for 1 h with 50 μg/ml gentamicin, and the medium was replaced with 25 μg/ml gentamicin. After incubation for 1 h or 24 h, macrophages were lysed with 0.5% Tween 20, and bacteria were collected, diluted in PBS, and plated onto LB agar supplemented with nalidixic acid.

RT-PCR. The extraction of total RNA from the spleen with TRIzol reagent (Invitrogen, Carlsbad, CA) was performed as described previously (49). For a quantitative analysis of mRNA levels, 500 ng of total RNA from each sample was reverse transcribed in a 50-μl volume (TaqMan reverse transcription [RT] reagent; Applied Biosystems), and 4 μl of cDNA was used for each real-time reaction. cDNA was amplified with the following primer sets for mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (forward primer 5′-AGGTCGG TGTGAACGGATTTG-3′ and reverse primer 5′-TGTAGACCATGTAGTTG AGGTCA-3′) (49) and interleukin-12 (IL-12)/IL-23p40 (*Il12b*) (forward primer 5′-GGAAGCACGGCAGCAGAATA-3′ and reverse primer 5′-AACTTGAGG GAGAAGTAGGAATGG-3′) (40) by using SYBR green PCR master mix (Applied Biosystems) and an ABI Prism 7900HT sequence detection system (Applied Biosystems). The data were analyzed by using the comparative threshold cycle (C_T) method (Applied Biosystems). Fold increases in cytokine expression in infected mice were calculated relative to the average level of the respective cytokine in control animals.

Statistical analysis. The statistical significance of differences between groups was determined by a Student's *t* test on normally distributed data. Data that were not normally distributed were logarithmically transformed before analysis by a Student's *t* test. Significant differences among treatment groups were determined by analysis of variance (ANOVA) followed by a Tukey honestly significant difference (HSD) test for pairwise comparisons, or for data with a non-Gaussian distribution, a Kruskal-Wallis nonparametric ANOVA was used. The GraphPad InStat program was used for statistical analysis. A *P* value of 0.05 was considered to be significant.

RESULTS

Establishment of a mouse model to study *S.* Typhimurium/*P. yoelii nigeriensis* coinfection. To investigate the mechanisms by which malaria increases the susceptibility to systemic *S.* Typhimurium infection in pediatric patients, we developed a mouse model of coinfection. For this work we selected inbred CBA/J mice for their resistance to both *S.* Typhimurium (45) and *P. yoelii nigeriensis* (17).

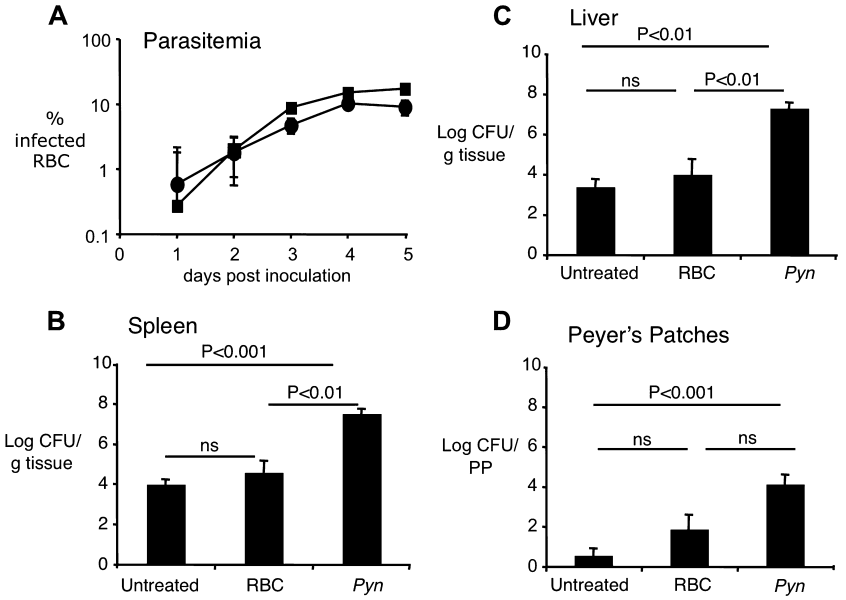

FIG. 2. Colonization of mice during infection with individual pathogens and during coinfection. (A) Parasitemia levels in mice infected with *P. yoelii nigeriensis* alone (squares) and during coinfection with *S.* Typhimurium (circles). Data represent means ± standard deviations (SD) for five mice. (B to D) Organ colonization by *S.* Typhimurium 5 days after i.g. infection of untreated mice, mice injected i.p. with naïve cells (RBC), or mice injected with red blood cells containing *P. yoelii nigeriensis* (*Pyn*). Numbers of CFU from the spleen (B), liver (C), and PP (D) are represented as the means ± standard errors of results from groups of 9 to 17 mice. Statistical significance was analyzed by using a Kruskal-Wallis nonparametric ANOVA followed by Dunn's multiple-comparisons test. *P* values for each pairwise comparison are shown. ns, not significant.

In order to determine whether simultaneous infection with *S.* Typhimurium and *P. yoelii nigeriensis* would affect parasitemia, mice were inoculated i.p. with *P. yoelii nigeriensis*-infected RBC followed by i.g. inoculation of *S.* Typhimurium or i.g. administration of LB broth (negative controls). Mice were monitored daily by enumerating the percentage of RBC that contained parasites (Fig. 2A). The results show that neither the onset of parasitemia nor the maximal parasitemia was affected by coinfection. To determine whether infection with *P. yoelii nigeriensis*-infected RBC by the i.p. route would exacerbate the bacterial colonization of spleen, liver, and Peyer's patches (PP), bacterial loads were determined 5 days after infection with both pathogens (Fig. 2B to D). Two groups of controls were included for these experiments: mice infected only with *S.* Typhimurium (untreated) and mice injected with parasite-free RBC and subsequently infected with *S.* Typhimurium (to control for the effects of the RBC injection). The numbers of *S.* Typhimurium cells recovered from the liver and spleen were increased by approximately 1,000-fold ($P < 0.01$) (Fig. 2B and C), with a trend for higher numbers of CFU in the PP (Fig. 2D) in coinfected mice than in the control groups. These data provided evidence that in our model, malaria resulted in a marked increase in bacterial recovery from spleen, liver, and PP. We concluded that our animal model was suitable for the further study of the mechanism(s) by which malaria increases susceptibility to systemic *S.* Typhimurium infection.

Coinfection with *S.* Typhimurium does not exacerbate spleen or liver pathology caused by *P. yoelii nigeriensis*. Based on the higher loads of *S.* Typhimurium in livers and spleens of coinfected mice, we determined whether coinfection would exacerbate tissue pathology in these organs. Grossly, splenomegaly was observed for mice infected with either *S.* Typhimurium or *P. yoelii nigeriensis.* However, despite the increased bacterial loads in coinfected mice, there was no increase in splenomegaly in coinfected mice compared to mice infected with *P. yoelii nigeriensis* alone (Fig. 3A). Microscopically, spleens of *P. yoelii nigeriensis*-infected mice exhibited a marked increase of reticuloendothelial cells of the red pulp. Multiple macrophages filled with hemozoin were observed (Fig. 3B). Spleens of *S.* Typhimurium-infected mice exhibited a mild neutrophil infiltrate in the red pulp (Fig. 3B). Both lesions were apparent in spleens of coinfected mice, with no apparent exacerbation of either lesion (Table 1).

Livers of *P. yoelii nigeriensis*-infected mice exhibited a marked adherence of monocytic cells and parasitized RBC (pRBC) to the vascular endothelium (Fig. 3B). A mild mononuclear cell infiltrate in the sinusoids and many Kupffer cells filled with hemozoin were observed. Livers of *S.* Typhimurium-infected mice exhibited multifocal abscess formation (Fig. 3B). Abscesses were small and randomly distributed, with no association to specific hepatic structures. Livers of coinfected mice exhibited lesions typical of both *S.* Typhimurium and malaria parasite infection, with no apparent increase in either the number or size of either type of lesion (Fig. 3B and Table 2).

Hemolytic anemia increases susceptibility to systemic *S.* Typhimurium infection. Clinical reports documenting that individuals with sickle cell disease are at an increased risk of developing systemic NTS infection (10, 28, 59, 60) suggest that hemolytic anemia may be a factor contributing to *S.* Typhimurium bacteremia. To test whether hemolytic anemia in the absence of malaria parasite infection could increase systemic loads of *S.* Typhimurium in our model, we injected rabbit anti-mouse RBC immunoglobulin (IgG) intraperitoneally, fol-

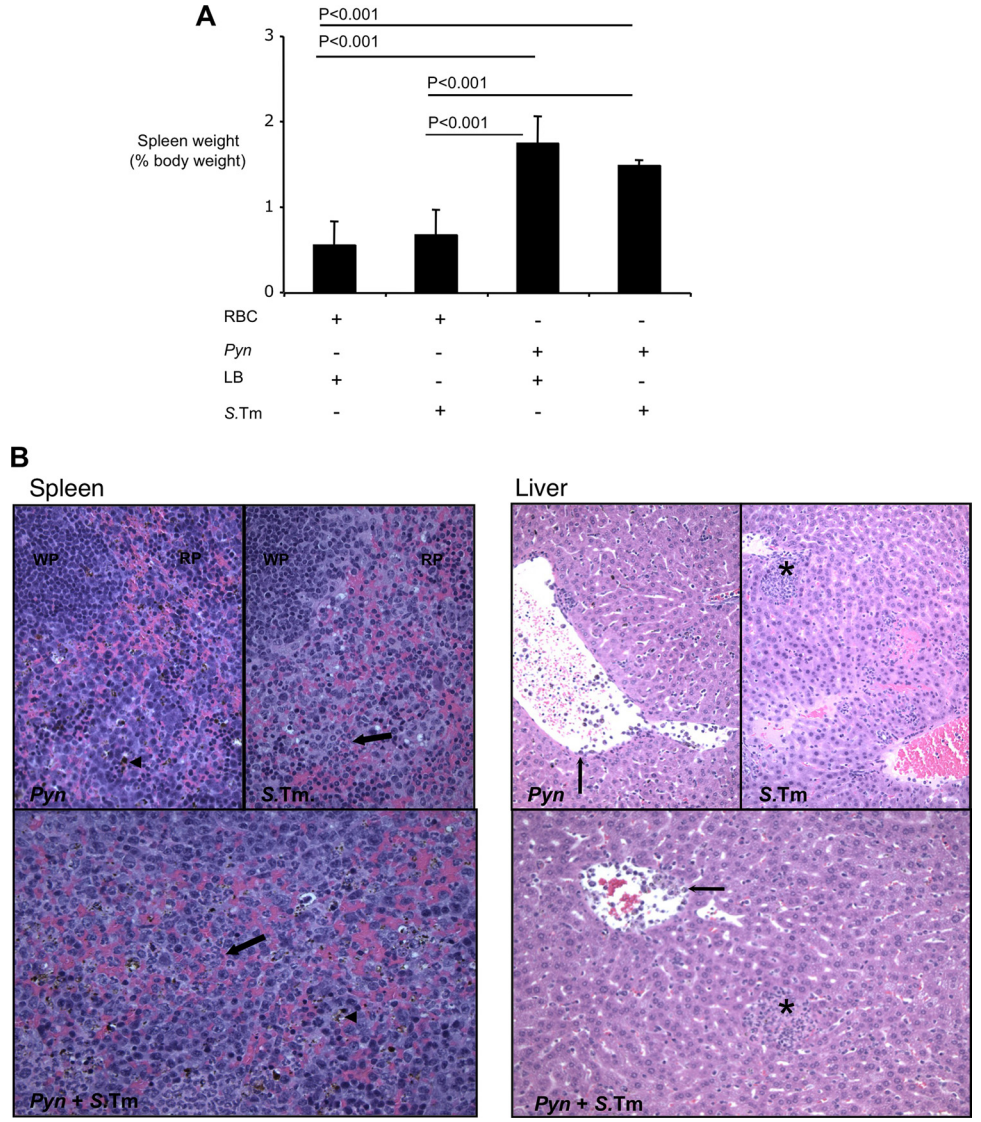

FIG. 3. Effect of *S.* Typhimurium infection on pathology in spleen and liver. (A) Mice were injected with naïve RBC or *P. yoelii nigeriensis*-infected RBC (*Pyn*) and subsequently gavaged with *S.* Typhimurium (*S.*Tm) or LB. On day 5 postinfection, the spleen weight was calculated as a percentage of the body weight. (B) Histopathological lesions in HE-stained sections of liver and spleen after infection with *P. yoelii nigeriensis* (magnification, ×200) or *S.* Typhimurium (magnification, ×200) or coinfection with *P. yoelii nigeriensis* and *S.* Typhimurium (magnification, ×400). (Left) Arrowheads point to splenic macrophages filled with hemozoin pigment. Thick arrows indicate neutrophil infiltrates in the red pulp (RP) after *S.* Typhimurium infection. (Right) Thin arrows show the adherence of monocytes on vascular endothelial cells. Asterisks indicate microabscess formation (arrow). Images are representative of groups of 5 mice. WP, white pulp.

lowed by infection with *S.* Typhimurium. Mice injected with nonimmune rabbit IgG followed by *S.* Typhimurium infection served as negative controls. The severity of anemia induced by the injection of rabbit anti-mouse RBC IgG was similar to that produced by *P. yoelii nigeriensis* infection, with an approximately 30% reduction in the concentration of erythrocytes in the blood after both treatments (Fig. 4). Five days after the induction of noninfectious hemolytic anemia, the recovery of *S.* Typhimurium from the spleen and liver was increased by approximately 10-fold (Fig. 5A and B). However, there were no significant differences in bacterial loads recovered from PP (Fig. 5C). The finding that the induction of the same degree of anemia by either malaria parasite infection or hemolysis resulted in more severe disseminated infection in the case of malaria parasite infection suggested that hemolytic anemia was a contributing factor rather than the sole factor responsible for the increased systemic loads of *S.* Typhimurium in mice with severe malaria. These observations raised the possibility that at least two independent mechanisms contributed to immunosuppression during malaria, including hemolytic anemia and an unknown *P. yoelii nigeriensis*-specific effect.

Erythrophagocytosis modestly reduces the ability of macrophages to control *S.* Typhimurium replication. Macrophages in the spleen and liver participate in the clearance of damaged RBC, and it was suggested previously that the increased uptake of parasitized RBC by the spleen may reduce the ability of

TABLE 1. Mean histopathology scores of different lesions observed in spleens of infected mice[a]

Group	Mean histopathology score			
	Increase of histiocytes in red pulp	Increase of neutrophils in red pulp	Lymphoid hyperplasia	Intracellular pigment
Control ($n = 15$)	0[b]	0.27[b]	0.3[b]	0[b]
P. yoelii nigeriensis ($n = 5$)	1.2[c]	0.4[b,c]	2.4[b,c]	2.2[c]
S. Typhimurium ($n = 20$)	0[b]	1.4[c]	0.7[b,d]	0[b]
Coinfection ($n = 5$)	1.2[c]	1.8[b,c]	2.2[c,d]	2.2[c]

[a] Histopathology scores are as follows: 0, absent; 1, mild; 2, moderate; 3, severe.
[b] Different letters in the same column are statistically different ($P < 0.05$ by Kruskal-Wallis test).
[c] Different letters in the same column are statistically different ($P < 0.05$ by Kruskal-Wallis test).
[d] Different letters in the same column are statistically different ($P < 0.05$ by Kruskal-Wallis test).

macrophages to control bacterial replication during malaria infection (22). To determine whether a reduced bactericidal activity of macrophages could account for the immunosuppressive effects of severe anemia, J774.A1 macrophages were treated with antibody-opsonized RBC, nonopsonized RBC, or no RBC prior to infection with *S.* Typhimurium (Fig. 6). Only treatment with opsonized RBC significantly increased the replication of *S.* Typhimurium. However, the recovery of *S.* Typhimurium from macrophages that had internalized opsonized RBC was increased only 2-fold relative to that of macrophages exposed to untreated RBC by 24 h. While red blood cell uptake did reduce the ability of macrophages to restrict bacterial replication, the effects were modest compared to the differences in bacterial recovery observed *in vivo* for mice with noninfectious anemia (Fig. 5). Thus, it appears likely that hemolytic anemia may cause additional immune defects *in vivo* that reduce the host's ability to restrict the systemic spread and/or replication of *S.* Typhimurium.

TABLE 2. Mean histopathology scores of different lesions observed in livers of infected mice[a]

Group	Mean histopathology score			
	Sinusoidal leukocytosis	Microabscess	Endothelial adherence of leukocytes	Intracellular pigment
Control ($n = 15$)	0.1[b]	0.07[b]	0[b]	0[b]
P. yoelii nigeriensis ($n = 5$)	1.2[c]	0.6[b,c]	1.6[c]	2.0[c]
S. Typhimurium ($n = 20$)	0.55[b,c]	1.15[c]	0.1[b]	0[b]
Coinfection ($n = 5$)	1.4[c]	1.6[c]	2.0[c]	1.8[c]

[a] Histopathology scores are as follows: 0, absent; 1, mild; 2, moderate; 3, severe.
[b] Different letters in the same column are statistically different ($P < 0.05$ by Kruskal-Wallis test).
[c] Different letters in the same column are statistically different ($P < 0.05$ by Kruskal-Wallis test).

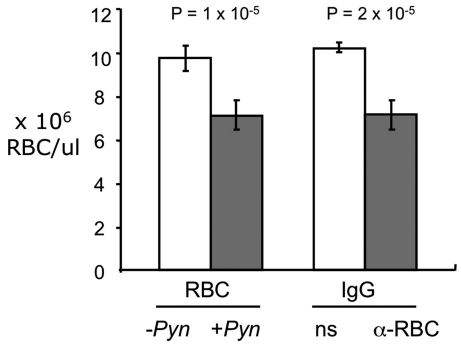

FIG. 4. *P. yoelii nigeriensis* infection and antibody-mediated hemolysis elicit similar degrees of anemia. Mice were inoculated i.p. with 5×10^6 RBC containing *P. yoelii nigeriensis* (+*Pyn*) or with an equivalent amount of uninfected blood (−*Pyn*) (uninfected controls). To induce noninfectious hemolytic anemia, mice were injected i.p. with rabbit anti-mouse RBC IgG (α-RBC) or nonspecific rabbit IgG (ns) (controls). Erythrocyte counts were determined at day 5 after infection or treatment. Data shown are means ± SD for 5 mice per treatment group. Differences between treatment groups and their respective controls were analyzed by a Student's *t* test. Data shown are from a representative experiment that was performed twice. *P* values are shown above each data set.

***Plasmodium yoelii nigeriensis* infection, but not noninfectious anemia, leads to reduced serum IL-12 levels during *S.* Typhimurium infection.** IL-12 is essential for inducing protective immunity against *S.* Typhimurium (23, 24, 27, 32) and *Plasmodium* spp. (29, 30, 52). Children with severe malaria have reduced serum IL-12 levels (26, 31). Since splenic dendritic cells and macrophages synthesize IL-12 inducibly upon infection with *S.* Typhimurium (53), we tested whether hemolytic anemia or malaria parasite infection would affect levels of IL-12 in the serum (Fig. 7). Coinfection with *P. yoelii nigeriensis* led to a significant reduction of IL-12p70 levels in the serum of *S.* Typhimurium-infected mice compared to mice infected only with *S.* Typhimurium (Fig. 7A). In contrast, the induction of hemolytic anemia led to a 2-fold increase in serum IL-12p70 levels in *S.* Typhimurium-infected mice (Fig. 7B), suggesting a malaria parasite-specific blunting of the IL-12 response to *S.* Typhimurium. Thus, since mice with malaria parasite infection have both parasites and anemia, it is possible that anemia and malaria parasites individually may exert opposite effects on IL-12 responses to *S.* Typhimurium, the sum of which is a moderate increase in the IL-12 level that was observed for the malaria parasite-infected mice.

DISCUSSION

Epidemiological studies have reported that NTS are among the leading causes of invasive bacterial infection in children in sub-Saharan Africa. The main risk factors for developing NTS in Africa include malnutrition, HIV infection, and severe malaria. (3, 5, 6, 14, 16, 25, 34, 57). Concurrent infections with malaria and NTS have been reported to result in the systemic dissemination of bacteria, which in turn leads to increased morbidity for children under the age of 5 years.

Despite the magnitude of this public health problem in sub-Saharan Africa, there is currently only one previous report in the literature investigating experimental malaria NTS coinfec-

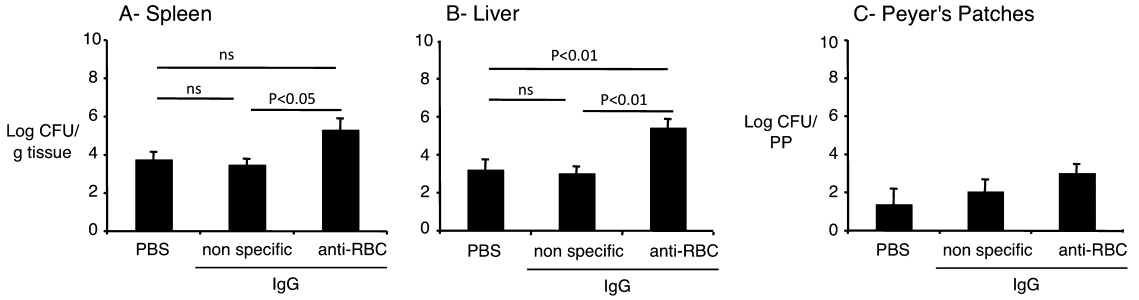

FIG. 5. Hemolytic anemia increases systemic *S.* Typhimurium colonization. Shown are data for organ colonization by *S.* Typhimurium 5 days after i.g. inoculation with bacteria and i.p. inoculation with either PBS, nonspecific IgG, or anti-erythrocyte IgG. Log CFU from the spleen (A), liver (B), and PP (C) are represented as means ± standard errors of logarithmically transformed data from 6 mice. Statistical significance was analyzed by using ANOVA followed by a Tukey's HSD test. *P* values for each pairwise comparison are shown. ns, not significant.

tion. In that study, male Swiss mice inoculated with *Plasmodium berghei* were challenged intravenously with *S.* Typhimurium (22). Here we established a model in which *P. yoelii nigeriensis*-infected mice were challenged with *S.* Typhimurium by the natural (oral) route of infection. We showed that *P. yoelii nigeriensis* infection reproduced severe hemolytic anemia, an important clinical feature that has been associated with NTS bacteremia (15).

Our data showed that an increased susceptibility of malaria parasite-infected mice to *S.* Typhimurium was due in part to hemolytic anemia. Experimentally induced hemolytic anemia was previously implicated in an increased susceptibility of mice to *S.* Typhimurium (20, 21). This notion is also supported by epidemiological studies, which showed that anemia from sickle cell disease is associated with an increased susceptibility to disseminated NTS infection (10, 60). Based on an increased recovery of *S.* Typhimurium from macrophages after erythrophagocytosis, it was proposed that hemolytic anemia increases susceptibility to *S.* Typhimurium infection by reducing the microbicidal activity of macrophages (11). While we were able to reproduce this phenotype, the magnitude of the increase in *S.* Typhimurium recovery from erythrophagocytic macrophages was modest (2-fold), making it difficult to conclude that erythrophagocytosis is the sole mechanism by which hemolytic anemia contributes to bacteremia. Previous *in vitro* data suggesting that the uptake of RBC by macrophages inhibited their bactericidal activity imply that both *S.* Typhimurium and parasitized erythrocytes would be within the same cell. If this were the case, then one factor contributing to the modest increase in bacterial multiplication could be an increased availability of iron and other nutrients to *S.* Typhimurium inhabiting the same cell. However, an *in vivo* study using *S.* Typhimurium to study the function of the splenic marginal zone during malaria infection showed that the bacteria localized to the marginal zone, while erythrocytes parasitized with the murine malaria agent *P. chabaudi adami* localized to the red pulp (61). These results suggest a cytokine-mediated suppression of bactericidal or bacteriostatic functions by malaria parasite infection rather than a direct effect on individual coinfected cells.

In our model, malaria parasite infection reduced circulating IL-12p70 levels in response to *S.* Typhimurium infection. IL-12 is known to play an important role in response to *S.* Typhimurium, and the antibody-mediated neutralization of IL-12 in

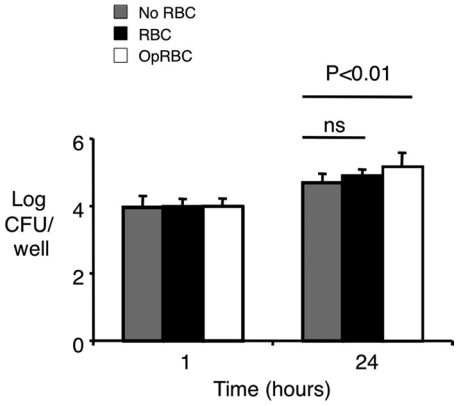

FIG. 6. Effect of RBC uptake on intracellular replication of *S.* Typhimurium. Results are represented as means ± standard deviations from three independent experiments. Opsonized or nonopsonized sheep RBC were incubated with J774 macrophages at a ratio of 20:1 for 2 h at 37°C. Control macrophages were not treated with RBC. Macrophages were then infected with *S.* Typhimurium (MOI of 5) and lysed 1 and 24 h after infection to enumerate CFU. Statistical significance was determined by ANOVA followed by a Tukey's HSD test. ns, not significant.

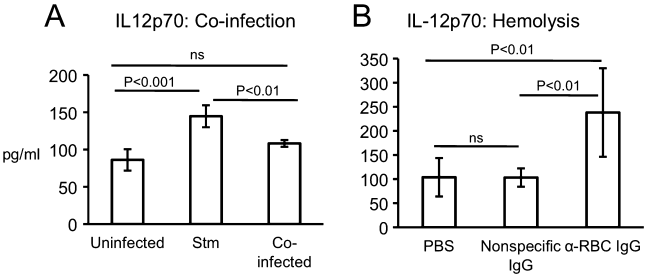

FIG. 7. Malaria parasite infection, but not hemolytic anemia, blunts the IL-12 response to *S.* Typhimurium infection. IL-12p70 in serum was assayed by enzyme-linked immunosorbent assay (ELISA) at 5 days after infection. (A) Mice were singly infected with *S.* Typhimurium (Stm) or coinfected with *P. yoelii nigeriensis.* (B) Mice were mock treated with PBS (untreated controls) or inoculated with nonspecific IgG or with anti-RBC IgG to induce anemia. Results are the means ± standard deviations for 3 to 6 mice per treatment group. Statistical significance was analyzed by ANOVA followed by a Tukey's HSD test. *P* values for each pairwise comparison are indicated. ns, not significant.

mice exacerbates infection (32). Furthermore, clinical data have demonstrated an association of genetic defects in the IL-12/gamma interferon axis with an increased risk of developing systemic NTS infections (27, 41, 54). Thus, a reduction in levels of IL-12 in coinfected mice suggests an underlying effect of malaria parasite infection on the ability of phagocytic cells, such as dendritic cells and macrophages, to recognize and respond appropriately to pathogens. Immunosuppressive effects of malaria have been observed for both infected individuals and infection models. For example, severe childhood malaria is an important underlying factor in the development of Burkitt's lymphoma caused by Epstein-Barr virus (48). Furthermore, in a study from 1965, subjects who were experimentally infected with malaria parasites were resistant to the endotoxic effects of subsequently injected lipopolysaccharide (LPS) (50). One cell population that was shown to have a reduced responsiveness as a result of malaria parasite infection is dendritic cells, which in mice produce less IL-12 in response to LPS after *P. yoelii* infection (35) and in humans undergo reduced maturation after taking up *P. falciparum*-infected erythrocytes (56). It was proposed previously that the reduced responsiveness of dendritic cells to Toll-like receptor (TLR) agonists may prevent excessive inflammation and tissue damage during malaria (43); however, the cost of this may be an increased susceptibility to other infections.

Reduced IL-12 levels during malaria parasite infection may also impact the development of anemia. Mohan and Stevenson (33) previously demonstrated an IL-12 deficiency during *Plasmodium chabaudi* infection that had a cause-and-effect relationship with a dysregulation of erythropoiesis and, therefore, was a likely contributor to severe anemia in mice. These data are consistent with data from clinical reports showing that low serum levels of IL-12 during pediatric malaria are correlated with severe anemia (31, 42).

While *P. yoelii nigeriensis* infection and treatment with anti-RBC IgG induced similar degrees of anemia in mice, *S.* Typhimurium loads in spleen and liver were approximately 100-fold greater during *P. yoelii nigeriensis* infection. These data suggested that in addition to anemia, malaria parasite-specific factors contributed prominently to the increased systemic dissemination and/or replication of *S.* Typhimurium in mice with malaria. The picture emerging from this work establishing a *P. yoelii*/*S.* Typhimurium coinfection model is that multiple distinct mechanisms, which contribute to immunosuppression during malaria, increase susceptibility to systemic NTS infection.

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