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The mucosal inflammatory response to non-typhoidal *Salmonella* in the intestine is blunted by IL-10 during concurrent malaria parasite infection

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Coinfection can markedly alter the response to a pathogen, thereby changing its clinical presentation. For example, non-typhoidal *Salmonella* (NTS) serotypes are associated with gastroenteritis in immunocompetent individuals. In contrast, individuals with severe pediatric malaria can develop bacteremic infections with NTS, during which symptoms of gastroenteritis are commonly absent. Here we report that, in both a ligated ileal loop model and a mouse colitis model, malaria parasites caused a global suppression of gut inflammatory responses and blunted the neutrophil influx that is characteristic of NTS infection. Further, malaria parasite infection led to increased recovery of *Salmonella enterica* serotype Typhimurium from the draining mesenteric lymph node (MLN) of mice. In the mouse colitis model, blunted intestinal inflammation during NTS infection was independent of anemia but instead required parasite-induced synthesis of interleukin (IL)-10. Blocking of IL-10 in coinfected mice reduced dissemination of *S*. Typhimurium to the MLN, suggesting that induction of IL-10 contributes to development of disseminated infection. Thus IL-10 produced during the immune response to malaria in this model contributes to suppression of mucosal inflammatory responses to invasive NTS, which may contribute to differences in the clinical presentation of NTS infection in the setting of malaria.

INTRODUCTION

In immunocompetent individuals, non-typhoidal *Salmonella* (NTS) serotypes are associated with gastroenteritis, a localized infection with low mortality that manifests as diarrhea, vomiting, and intestinal cramping. However, immunocompromised individuals can develop a life-threatening NTS bacteremia.[1] Epidemiological associations suggest that the most common immunocompromising conditions predisposing to pediatric NTS bacteremia in sub-Saharan Africa are malnutrition and severe malaria.[1–4] The magnitude of the public health problem posed by NTS bacteremia is little publicized, yet this condition contributes considerably to morbidity and mortality throughout Africa.[4] For example, NTS, specifically *Salmonella enterica* serotype Typhimurium and *S. enterica* serotype Enteritidis, are currently the most common blood isolates from children[2,3] and the second most common cause of pediatric meningitis in Malawi,[5] resulting in mortality rates exceeding 20%, despite antibiotic therapy.[6] A factor complicating treatment of invasive NTS is the high prevalence of multidrug resistance.[7–10] Although the occurrence of NTS bacteremia in pediatric malaria patients is well documented, little is known about immunological mechanisms that alter the host pathogen interaction during coinfection.

The intestinal pathology of immunocompetent individuals with NTS gastroenteritis is characterized by inflammatory infiltrates that are dominated by neutrophils.[11] This massive influx of neutrophils has an important role in producing signs of gastroenteritis. For example, CD18-deficient animals, whose

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neutrophils are unable to extravasate from the circulation, displayed markedly decreased intestinal pathology and fluid secretion in response to NTS infection.[12] Interestingly, clinical and epidemiological studies of NTS bacteremia in children with malaria report a lack of association with symptoms of gastroenteritis.[13,14] These findings suggested that malaria may affect mucosal immune responses to NTS infection. To test this hypothesis, we utilized two coinfection models, rhesus macaques and mice, to investigate the intestinal inflammatory responses during NTS malaria coinfection. Our results identified a novel mechanism by which malaria alters host responses to NTS infection.

RESULTS

Malaria parasite infection blunts the intestinal response to *Salmonella* Typhimurium

As the clinical course of non-typhoidal *S.* Typhimurium infection is generally acute, whereas malaria is associated with more protracted illness, we reasoned that in endemic areas individuals would be most likely to become infected with NTS after contracting malaria. Thus, an assumption underlying our models is that NTS infection is subsequent to malaria. As *Plasmodium falciparum* is the most common cause of malaria in sub-Saharan Africa, we used a non-human primate model of falciparum malaria to study whether underlying malaria affects the initial mucosal response to a secondary infection with *S.* Typhimurium. For this work, rhesus macaques (*Macaca mulatta)* were infected with the simian malaria parasite *Plasmodium fragile*, which causes clinical disease in macaques that is similar to falciparum malaria in humans.[15,16] To study the effect of underlying malaria parasite infection on the initial mucosal inflammatory response to *S.* Typhimurium infection in the intestine, we used a ligated ileal loop model.[17] This model allowed us to compare early mucosal responses of uninfected (control) macaques to those of macaques infected with *P. fragile* (**Figure 1**). For these experiments, macaques ($n = 4$) were

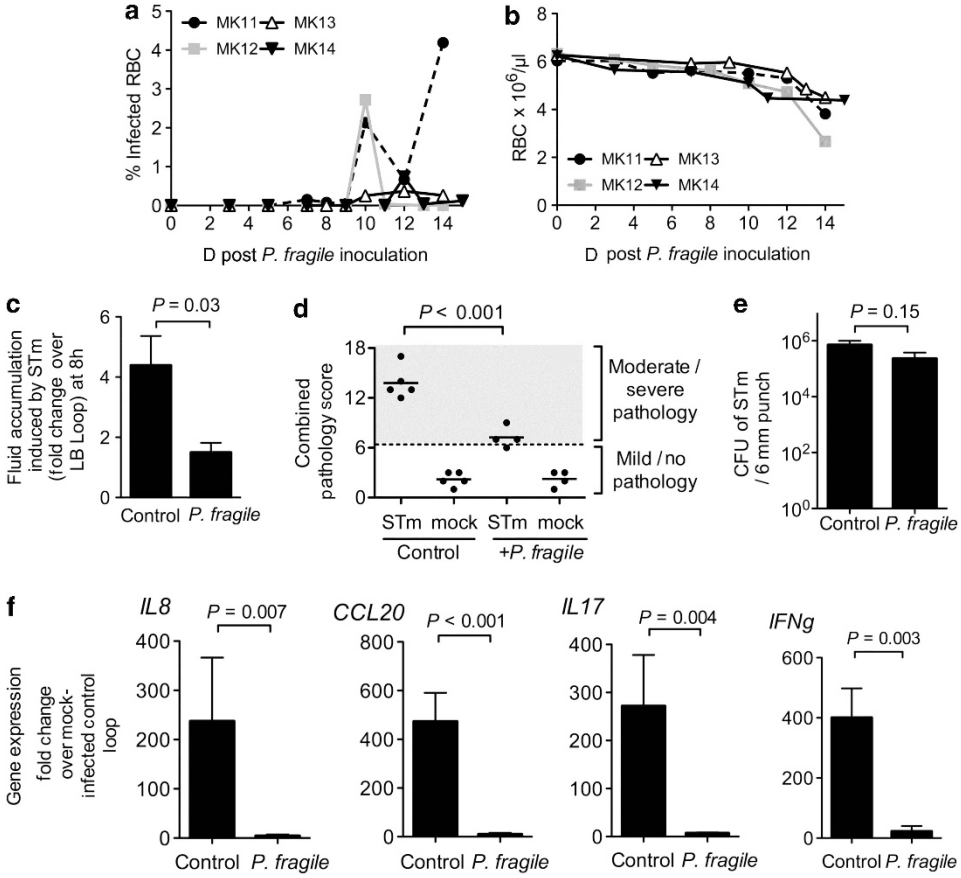

Figure 1 Underlying infection with *Plasmodium fragile* blunts intestinal inflammation elicited by *S.* Typhimurium in rhesus macaques. (**a**) Time course of parasitemia in four animals (MK11-14) after inoculation with blood-stage *P. fragile*. (**b**) Concentration of circulating red blood cells (RBCs) over the course of the experiment. (**c**) Fluid accumulation in the lumen of ligated ileal loops, 8 h after inoculation with *S.* Typhimurium. Responses of macaques inoculated with *P. fragile* ($n = 4$) were compared with control (uninfected) animals ($n = 5$). Results are expressed as the ratio of fluid accumulation in a loop inoculated with *S.* Typhimurium to fluid in a control loop injected with Luria-Bertani (LB) broth in the same animal. (**d**) Blinded histopathology scoring of ileal mucosa at 8 h after inoculation of ligated ileal loops. Individual components of the combined pathology scores are shown in **Supplementary Figure S1**. (**e**) Tissue-associated bacteria at 8 h after inoculation of ligated ileal loops. (**f**) Expression of proinflammatory cytokines in ileal mucosa of control or *P. fragile*-infected animals at 5 h after inoculation with *S.* Typhimurium ($n = 4$). Data are expressed as the fold change in the expression of *S.* Typhimurium-inoculated loop over mock (LB)-injected control loop for each animal and represent the mean ± s.e.m. of four animals per group. CFU, colony-forming units; CCL, C-C motif chemokine ligand; IFN, interferon; IL, interleukin.

inoculated intravenously with blood-stage *P. fragile*. As in a subset of animals *P. fragile* can cause lethal infection,[18] we monitored parasitemia closely and treated animals with a subcurative dose of quinine sulfate for 2 consecutive days when parasitemia rose above 0.5%. As shown in **Figure 1a**, all the four animals developed maximal parasitemias between 10 and 12 days after infection, which declined after quinine sulfate treatment. One animal (MK11) relapsed with high parasitemia after treatment with quinine sulfate. Peak parasitemia levels ranged from 1% to 4% in three animals, with the fourth developing only low (0.4%) parasitemia. However, it should be kept in mind that, as *P. fragile* sequesters on vascular endothelium, the total body parasite loads may not be reflected in blood parasite levels. Progressive decreases in circulating red blood cells (RBCs) after *P. fragile* infection were noted, which ranged from 29% to 57% (**Figure 1b**).

At 14-15 days after inoculation with *P. fragile*, ligated ileal loop surgery was performed, as described in the Materials and Methods. Loops were inoculated with either the wild-type (WT) NTS strain *S.* Typhimurium IR715 suspended in lysogeny broth (LB) or with sterile LB as a control and were sampled at 2, 5, and 8 h for evaluation of inflammatory responses. These responses were compared with those of uninfected control rhesus macaques that underwent the same ligated ileal loop procedure.[17] *P. fragile*-infected animals developed significantly less fluid accumulation in the ileal lumen (a surrogate marker for diarrhea) compared with controls at 8 h after inoculation (**Figure 1c**). In both control and *P. fragile*-inoculated macaques, *S.* Typhimurium infection elicited inflammatory changes, including neutrophil influx, hemorrhage, villus blunting, and epithelial loss in the ileal mucosa at 8 h, as evidenced by blinded histopathology scoring; however, these responses were significantly diminished in the *P. fragile*-infected group (**Figure 1d** and see **Supplementary Figure S1** online). The reduced inflammatory response in the *P. fragile*-infected animals did not appear to result from differences in numbers of tissue-associated bacteria, as similar numbers of bacteria were recovered from samples of ileal mucosa at 8 h (**Figure 1e**). As a second line of evidence for differences in inflammatory responses to *S.* Typhimurium in individuals with malaria, we assayed expression of proinflammatory cytokines at 5 h, an earlier time point at which no difference in fluid accumulation was yet evident (not shown). In control animals, as expected, *S.* Typhimurium induced a robust proinflammatory cytokine response. In contrast, this response was significantly attenuated in the *P. fragile*-infected animals (**Figure 1f**). These results suggested that underlying *P. fragile* infection blunted mucosal inflammatory responses to *S.* Typhimurium.

In order to identify mechanisms underlying the blunted mucosal responses to *S.* Typhimurium during malaria that were observed in macaques, we used a mouse coinfection model.[19] One drawback of the mouse for studying intestinal inflammation resulting from NTS infection is that, unlike in humans and non-human primates, signs of gastroenteritis do not readily develop. To overcome this limitation, we used the mouse colitis model, in which intestinal inflammation is promoted via treatment with streptomycin 1 day before infection with the *S.* Typhimurium.[20] To study the effects of severe malaria on mucosal responses to NTS, we used a mouse strain (CBA) that develops a non-lethal infection with either NTS or *Plasmodium yoelii nigeriensis* (*P. yoelii*), a rodent malaria parasite. Mice were inoculated intraperitoneally with blood-stage *P. yoelii*, and peak parasitemia was allowed to develop before inoculation with the *S.* Typhimurium strain IR715 at day 10 (**Figure 2a**). *P. yoelii*-infected mice exhibited a reduction in erythrocyte concentration by day 6, which progressed to significant anemia by day 10 (**Figure 2a**). Coinfected mice developed increased morbidity by 4 days after inoculation of *S.* Typhimurium (14 days after parasite infection), as evidenced by accelerated weight loss compared with mice infected individually with either pathogen (see **Supplementary Figure S2A**). The increased morbidity observed with coinfection mimics clinical features reported for pediatric malaria/NTS coinfections.[13]

As expected, *S.* Typhimurium infection induced a robust inflammatory response in the mouse colitis model, with submucosal edema, epithelial damage, and abundant exudation of neutrophils into the intestinal lumen at 48 h after *S.* Typhimurium infection (**Figure 2b–d**), and this effect was evident as early as 24 h (data not shown). In contrast, inoculation of CBA mice with *P. yoelii* alone was not associated with overt pathological changes to the intestinal mucosa relative to mock-infected control animals at 12 days after infection (**Figure 2b,c**). The inflammatory response associated with *S.* Typhimurium infection was significantly blunted at both 24 and 48 h in mice that were coinfected with *P. yoelii*, with a striking decrease in infiltration of neutrophils into the intestinal mucosa, submucosa, and lumen of coinfected mice (**Figure 2b–d**, see **Supplementary Figure S2B**, **S2C**, and data not shown). Despite a slight increase in intestinal colonization with *S.* Typhimurium in the coinfected mice, as shown by determination of bacterial numbers in the lumen of the colon (**Figure 2e**), pathological changes in the intestinal mucosa were reduced during coinfection (**Figure 2b,c** and see **Supplementary Figure S2B**).

Genomic analysis of epidemic *S.* Typhimurium isolates from Kenya and Malawi provided evidence of genome degradation affecting genes involved in virulence, which raised the possibility that epidemic *S.* Typhimurium isolates from Africa might be less able to elicit intestinal inflammation.[7] To test this hypothesis in our model, we utilized a sequenced *S.* Typhimurium bacteremia isolate from Malawi, D23580,[7] for use in parallel infection studies. In a manner that was analogous to our results with the North American *S.* Typhimurium isolate (ATCC14028), the isolate from Malawi (D23580) elicited inflammation in the mouse colitis model that was blunted by coinfection with *P. yoelii* (see **Supplementary Figure S2C–E**). The finding that both a North American and a Malawian *S.* Typhimurium bloodstream isolate elicited intestinal inflammation that was blunted by malaria parasite infection suggested that the Malawian strain was not deficient *per se* in eliciting intestinal inflammation. Instead, these data affirmed that malaria coinfection could

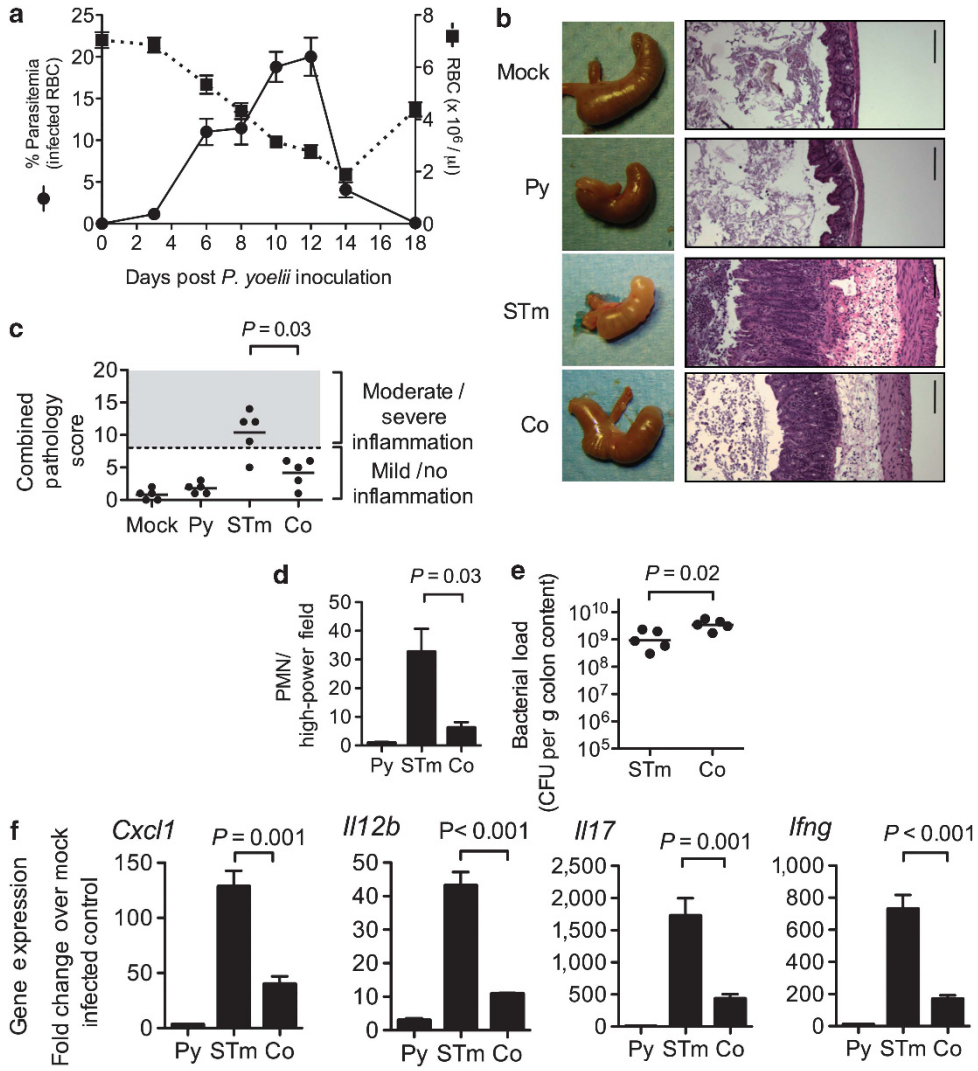

Figure 2 Effect of malaria parasite infection in a mouse colitis model. CBA mice were inoculated intraperitoneally with blood-stage *P. yoelii*. Control mice received an equivalent injection of red blood cells (RBCs) from uninfected mice. All mice were treated with streptomycin on day 9 followed by inoculation with *S.* Typhimurium (or an equivalent volume of Luria Bertani broth) on day 10. (**a**) Time course of parasitemia (left y axis) and development of anemia (right y axis) in coinfection studies. *S.* Typhimurium coinfection had no effect on the kinetics or level of *P. yoelii* parasitemia (not shown). Data represent the mean ± s.e.m. of four mice. (**b**) Gross appearance of the cecum at 48 h after *S.* Typhimurium infection in mock-treated mice, mice inoculated with *P. yoelii* only (*Py*), mice inoculated with *S.* Typhimurium only (*S*Tm), or coinfected mice (Co). At the right, a representative hematoxylin and eosin–stained section of the cecal mucosa is shown for each group. Scale bar = 200 μm. (**c**) Blinded histopathology scoring of inflammatory changes in cecal tissue at 48 h after *S.* Typhimurium infection ($n = 5$). Horizontal bar represents the mean. Individual components of the combined pathology scores are shown in **Supplementary Figure S2**. (**d**) Infiltration of neutrophils in the intestinal tissue of mice at 48 h after *S.* Typhimurium infection ($n = 5$). Data represent the mean ± s.e.m. (**e**) Enumeration of *S.* Typhimurium in the cecal content of *S.* Typhimurium-infected (*S*Tm) mice or mice coinfected with *P. yoelii* and *S.* Typhimurium (Co). Each dot represents a single mouse, and the horizontal lime represents the geometric mean. (**f**) Confirmation of blunted *Cxcl1*, *Cxcl2*, *Ifng* and *Il17a* responses 48 h after *S.* Typhimurium infection by quantitative PCR. Data were normalized to *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) expression and are shown as fold increase in mRNA with respect to mock-infected control mice.

commonly alter inflammatory responses in the intestinal mucosa during *S.* Typhimurium infection.

At the transcriptional level, a global suppression of immune responses to NTS infection was observed in the cecal mucosa of coinfected mice: at 2 days after *S.* Typhimurium infection, expression levels of 19.8% of genes induced by *S.* Typhimurium infection were reduced to 0.5-fold or lower in the *P. yoelii* coinfected mice relative to levels observed in mock-infected mice (see **Supplementary Figure S2F** and **Supplementary Table S1**). These results were validated by quantitative real-time PCR for transcripts encoding the neutrophil chemoattractant keratinocyte (*Cxcl1*), Cxcl2/Mip2α (*Cxcl2*), interferon (IFN)-γ (*Ifng*), and interleukin (IL)-17 (*Il17a*) (**Figure 2f**), as well as several additional transcripts encoding proinflammatory cytokines (data not shown). Thus, while malaria parasite infection on its own did not affect expression of proinflammatory cytokines in the cecum at 12 days after infection, severe malaria blunted the initial stages of the intestinal inflammatory response to *S.* Typhimurium that are important for recruitment of inflammatory cells to the site of infection.

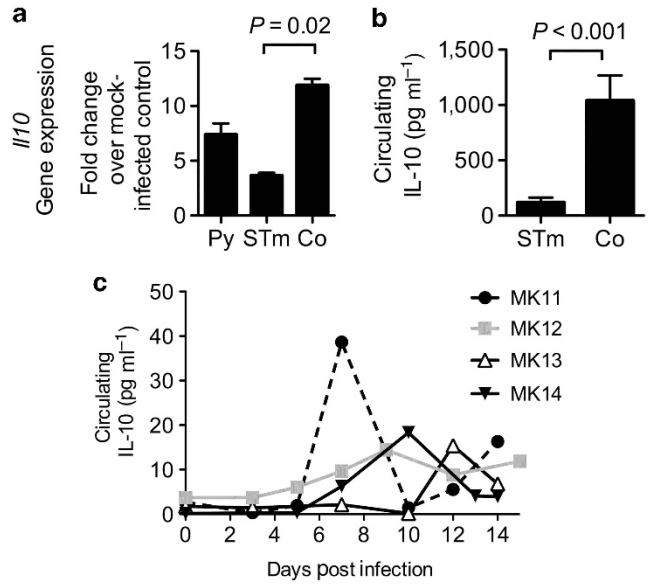

Figure 3 Induction of interleukin (IL)-10 by malaria parasite infection. (**a**) Induction of *Il10* in the cecum of coinfected mice at 48 h after *S.* Typhimurium infection. Data are normalized to *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) expression and are represented as mean fold increase in mRNA with respect to mock-infected control mice ($n = 5$). Differences in cytokine expression between groups were analyzed using analysis of variance with Tukey's posttest. (**b**) Levels of circulating IL-10 12 days after *P. yoelii* infection ($n = 5$). Data are represented as means and s.e.m. (**c**) Levels of circulating IL-10 in four rhesus macaques inoculated with *P. fragile*.

Malaria parasite infection leads to elevated IL-10 expression in mice and rhesus macaques

Severe malaria has been associated with induction of the immunoregulatory cytokine IL-10.[21–24] Locally, in the cecal mucosa of mice, both *P. yoelii* and *S.* Typhimurium infections induced expression of *Il10*, and this expression was significantly increased by coinfection (**Figure 3a**). Elevated levels of circulating IL-10 were also observed in the coinfected mice compared with mice infected with *S.* Typhimurium alone (**Figure 3b**). The *P. fragile*-infected macaques also exhibited elevated circulating IL-10, with maximal levels preceding the peak of parasitemia (**Figures 3c** and **1a**). However, at necropsy the local expression of IL-10 in the ileal mucosa did not differ significantly between *P. fragile*-infected and uninfected macaques (not shown). To determine whether severe anemia, which contributes to malaria severity and to risk of NTS bacteremia,[2,3] affected intestinal inflammation caused by *S.* Typhimurium or led to elevated *Il10* induction in the mouse model, we induced anemia by treatment with anti-RBC antibodies (see **Supplementary Figure S3**). Despite inducing anemia comparable to that induced by parasite infection (**Figure 2a**), anti-RBC treatment had no effect on intestinal inflammation or expression of proinflammatory cytokines induced by *S.* Typhimurium (see **Supplementary Figure S3B–D**). A statistically significant but quantitatively small increase in *Il10* expression was induced by anti-RBC treatment (see **Supplementary Figure S3E**), but this increase was notably lower than that induced in coinfected mice relative to mice infected only with *S.* Typhimurium (**Figure 3a**). Collectively, our data suggested that *S.* Typhimurium and *P. yoelii* coinfection led to elevated *Il10* transcription in the cecal mucosa independently of anemia, observations that are consistent with reports that severe malarial anemia is not associated with increased circulating IL-10.[25]

IL-10 induced by *P. yoelii* infection is required for blunting of mucosal inflammatory responses to *S.* Typhimurium and contributes to increased microbial translocation to the mesenteric lymph node (MLN)

To determine whether malaria parasite-induced IL-10 was required to blunt intestinal inflammatory responses to NTS, we performed a coinfection study in mice that are genetically deficient for IL-10. These mice have a C57BL/6 (*Slc11a1*$^{-/-}$) strain background, which differs from CBA mice both in susceptibility to lethal systemic *S.* Typhimurium infection[26] and in the kinetics of intestinal inflammation in the mouse colitis model[27] Therefore, we first established whether suppression of intestinal inflammation by malaria parasite infection could also be observed in C57BL/6 mice (**Figure 4**). Similar to results obtained with CBA mice (**Figure 2** and see **Supplementary Figure S2**), coinfection of C57BL/6 mice with *S.* Typhimurium and *P. yoelii* resulted in reduced inflammatory pathology associated with *S.* Typhimurium infection (**Figure 4a** and see **Supplementary Figure S4A**) and reduced influx of neutrophils into the cecum (**Figure 4b**). In contrast, in *Il10*$^{-/-}$ mice, *P. yoelii* coinfection did not blunt the inflammatory response to *S.* Typhimurium infection in the cecum, as measured by pathology score and tissue neutrophil counts (**Figure 4a,b** and see **Supplementary Figure S4B**). Further, in contrast to parental C57BL/6 mice, no significant reduction in the expression of *Cxcl1*, *Cxcl2*, *Ifng* or *Il17a* was observed in the cecal mucosa of *Il10*$^{-/-}$ mice at 2 days after inoculation with *S.* Typhimurium (**Figure 4c**). Although the C57BL/6 background allowed us to use *Il10*-deficient mice, a shortcoming of this model was that we were unable to follow coinfected mice beyond 3 days, as they developed lethal morbidity more rapidly than the CBA mice.

As our previous work demonstrated a decrease in intestinal barrier function during malaria and increased bacterial dissemination to the draining MLNs of mice,[28] we asked whether IL-10 elicited during malaria parasite infection could have a role in this phenotype. To this end, we determined the effect of IL-10 blocking in coinfected, streptomycin-treated CBA mice on bacterial loads in the draining MLNs of mice. Groups of mice infected with *S.* Typhimurium only or coinfected with *P. yoelii* and *S.* Typhimurium were treated with an IL-10 blocking antibody or with an isotype control (**Figure 5**). In the mice receiving the isotype control, significantly higher bacterial loads were detected at 2 days after *S.* Typhimurium infection in the MLN of coinfected mice, compared with mice infected with *S.* Typhimurium alone, confirming our previous report (**Figure 5a**).[28] In contrast, neutralization of IL-10 reduced recovery of *S.* Typhimurium

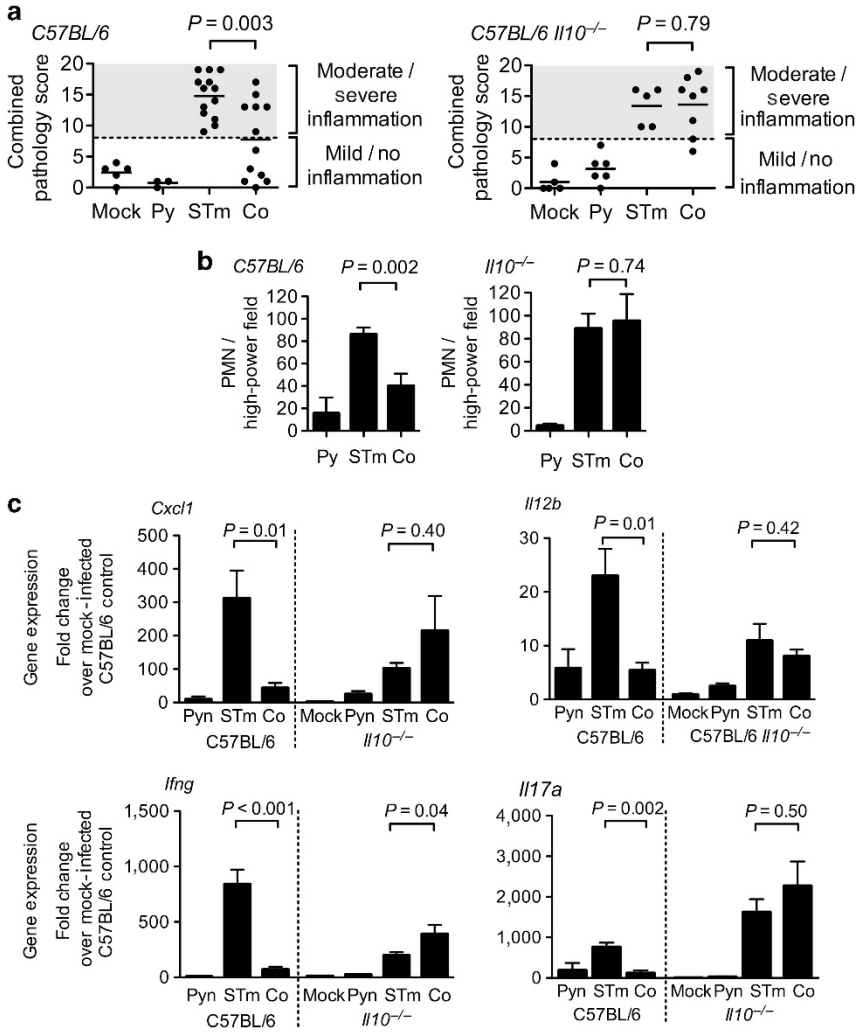

Figure 4 Interleukin (IL)-10 deficiency abrogates blunting of *S.* Typhimurium-induced intestinal inflammation by malaria parasites. (**a**) Blinded histopathology scoring of cecal tissue at 48 h after *S.* Typhimurium infection of C57BL/6 mice and isogenic mice deficient in IL-10. Bar represents the mean of 5–13 mice. *P*-value was determined by unpaired Student's *t*-test. Individual components of the combined pathology scores are shown in **Supplementary Figure S4**. (**b**) Infiltration of neutrophils in the intestinal tissue of mice at 48 h after *S.* Typhimurium infection. Data represent mean ± s.e.m. of 5-13 mice. *P*-value was determined by unpaired Student's *t*-test. (**c**) Expression of proinflammatory cytokines in the cecum of C57BL/6 or congenic *Il10*$^{-/-}$ mice were measured 48 h after *S.* Typhimurium infection by quantitative PCR ($n = 5$–8). Data were normalized to *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) expression and are shown as fold increase in mRNA with respect to mock-infected C57BL/6 controls. Bars represent mean ± s.e.m. *P*-values for C57BL/6 *Cxcl1* (C-X-C motif chemokine ligand 1) and C57BL/6 IL-10$^{-/-}$ *Il17* were determined by unpaired Student's *t*-test. Remaining *P*-values were determined by Mann–Whitney *U* test owing to non-Gaussian distribution. IFN, interferon; PMN, polymorphonuclear neutrophils.

from the MLN of coinfected mice to the level observed in mice infected with *S.* Typhimurium only, suggesting a contribution of IL-10 to the increased colonization of MLN during malaria parasite infection. Notably, IL-10 blocking had no effect on recovery of *S.* Typhimurium from the MLN of mice that were not infected with *P. yoelii*, suggesting that the effect of IL-10 on increased MLN colonization of *S.* Typhimurium is specific to malaria (**Figure 5a**). IL-10 blockade in coinfected mice partially increased inflammation in the cecum, as evidenced by increased expression of *Il12b* and *Il17a* in the cecum at 2 days after *S.* Typhimurium infection (**Figure 5b**). In contrast to what we observed in the IL-10 deficient C57BL/6 model, no significant effect of IL-10 blockade was observed on neutrophil influx in the cecum and on pathology score in the CBA mice at this time point (see **Supplementary Figure S5A,B**). This partial effect of IL-10 may be related to an increase in circulating IL-10 that was observed in the mice treated with the IL-10 blocking antibody (see **Supplementary Figure S5C**), or alternatively, it may indicate that additional mechanisms contribute to blunting of inflammation in the CBA mice. These results suggest that parasite-induced synthesis of IL-10 contributes to blunting of intestinal inflammation in the C57BL/6 model and, in the CBA model, to both blunting of IL-12p40 and IL-17, as well as an increase in extraintestinal *S.* Typhimurium during coinfection.

Taken together with the data presented above, our findings from animal models suggest that parasite-induced synthesis of IL-10 may contribute functionally to reduced gastroenteritis in

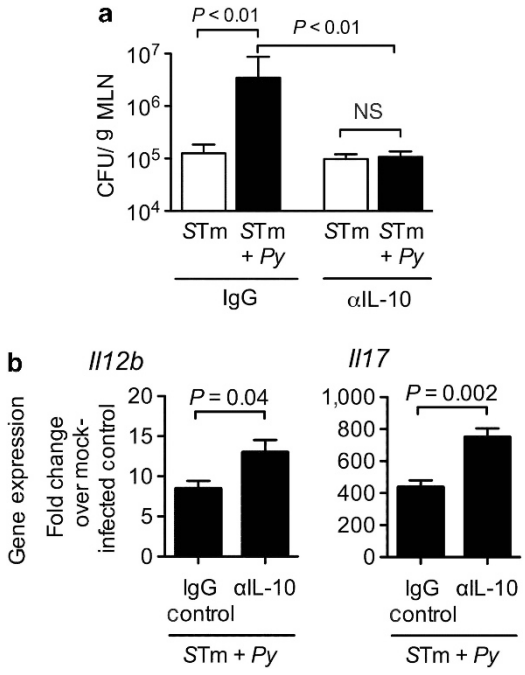

Figure 5 Blockade of *P. yoelii*-induced interleukin (IL)-10 in coinfected CBA mice increases inflammation and reduces dissemination of *S.* Typhimurium to the draining lymph node. CBA mice ($n = 5$) were infected with *S.* Typhimurium IR715 (*STm*) or coinfected with *S.* Typhimurium 12 days after infection with *P. yoelii* (*STm* + *Py*). (**a**) Recovery of *S.* Typhimurium from the mesenteric lymph node (MLN) of coinfected CBA mice treated with an IL-10 blocking antibody (αIL-10) or isotype control (immunoglobulin G (IgG)), at 2 days after inoculation with *S.* Typhimurium. Data are represented as mean ± s.e.m., and significance of differences was determined by a one-way analysis of variance on log-transformed data. Significance of differences between groups was determined using a Tukey's posttest. (**b**) Coinfected CBA mice were treated with αIL-10 or IgG, and expression of *Il12b* and *Il17a* in the cecum was assayed by quantitative reverse transcription–PCR. Data are represented as mean ± s.e.m., and significance of differences was determined by an unpaired *t*-test. CFU, colony-forming units.

malaria coinfected children, thereby leading to an altered clinical presentation of NTS infection.

DISCUSSION

Both acute and resolving malaria are associated with NTS bloodstream infections in children.[1–4,13] Severe anemia increases *S.* Typhimurium tissue load and lethality of infection in murine models,[19,29,30] and a recent report by Cunnington *et al.*,[31] demonstrated that severe malarial anemia, via induction of heme oxygenase, results in an inability of neutrophils to generate an oxidative burst. Importantly, this same defect in neutrophil oxidative killing activity was observed in children with acute or resolving *P. falciparum* malaria.[32,33] We did not observe an effect of anemia on intestinal inflammation in our model, as induction of anemia to levels found during *P. yoelii* infection of mice did not affect intestinal inflammation (see **Supplementary Figure S2**). In contrast, we found that the immunoregulatory environment induced by *P. yoelii* infection, specifically local production of IL-10, was responsible for reducing proinflammatory chemokine expression in the intestine. Thus, the chemotactic signals to attract neutrophils to the site of bacterial invasion at the intestinal mucosa appear to be defective during malaria. This reduced inflammation was observed both in mice, which had severe anemia, and in the macaque model, in which animals had more moderate RBC depletion. In patients with *Plasmodium vivax* malaria, a different neutrophil defect was noted, namely reduced migration toward a chemotactic gradient.[33] Thus, malaria may impact the function of neutrophils in multiple ways: via a hemolysis-dependent reduction in their oxidative killing capacity,[31] and by reducing their recruitment to sites of infection via reduction of both the chemotactic signal (**Figure 2**) and the migratory capacity[33] needed for neutrophil influx.

Our results suggested that during *P. yoelii* infection of mice, parasite-induced IL-10 was responsible for attenuation of inflammatory responses associated with protection against *S.* Typhimurium infection. IL-10 is known to have a critical role in preventing the development of excessive and potentially fatal pathology in experimental malaria models,[34–36] and the significance of these findings for human malaria is supported by observational data from malaria patients.[37,38] Further, in accord with our observations that anemia alone resulted in a comparatively smaller mean induction of cecal *Il10* expression relative to *P. yoelii* infection (see **Supplementary Figure S3E** and **Figure 3a**), other studies have reported stronger associations of elevated circulating IL-10 with uncomplicated malaria and cerebral malaria than with severe malaria anemia.[39,40] However, it is not clear from this previous work whether data for circulating IL-10 from a single blood draw are representative of IL-10 responses of the intestine, for example, that responds to invasive *S.* Typhimurium infection. In our *P. fragile*-infected rhesus macaques, circulating IL-10 was highly variable over the course of infection (**Figure 3c**), suggesting that this may also be the case in human malaria. Thus, additional studies will be needed to determine whether patterns of circulating IL-10 levels reflect functional local biology of IL-10 in tissues that are critical for host defense against secondary infections.

Here we show that in both C57BL/6 and CBA mice IL-10 production contributed to differing degrees to *P. yoelii*-mediated attenuation of intestinal inflammation caused by *S.* Typhimurium infection. Several cell types at systemic sites, including $CD4^+$ T cells, $CD19^+$ B cells, $CD11b^{high}Ly6C^+$ inflammatory monocytes, immature dendritic cells, and different populations of $CD4^+$ T cells, including effector Th1 cells and Treg cell populations, have been shown to produce IL-10 in murine malaria models and in humans (reviewed in Freitas do Rosario and Langhorne[41]). However, little is known about populations of immune cells in the intestine that might produce IL-10 during malaria. IL-10 produced during malaria parasite infection could suppress intestinal responses at multiple points. Given that IL-10 suppresses responsiveness of dendritic cells and macrophages to Toll-like receptor (TLR) ligands,[42] the ability of these mucosal cells to respond to invasive *S.* Typhimurium may be diminished. An early study on patients with *P. vivax* malaria demonstrated that these patients had a reduced febrile response to an injected *Salmonella* endotoxin preparation, suggesting a reduced responsiveness of these patients

to *Salmonella* pathogen–associated molecular patterns.[43] This reduced responsiveness could occur at the level of phagocytic cells, as both maturation of dendritic cells and their ability to activate T cells in response to treatment with TLR4 or TLR9 ligands is inhibited by malaria parasite infection.[44–46] Further, TLR signaling via Myd88 (myeloid differentiation primary response gene 88) is important for induction of the inflammatory response to *S.* Typhimurium in the mouse colitis model.[47,48] Thus, while previous studies confirmed suppression of systemic immune responses during malaria, our results are consistent with an IL-10-dependent suppression of mucosal inflammatory responses to invasive *Salmonella*.

In addition to blunting of intestinal inflammation, malaria parasite–induced IL-10 also promoted increased localization of *S.* Typhimurium to the draining MLNs of mice. *S.* Typhimurium, being an invasive organism, is able to disseminate to the draining lymph node on its own; however, malaria parasite infection promoted increased bacterial loads at this site (**Figure 5** and Chau *et al.*[28]). Multiple mechanisms could contribute to increased recovery of *S.* Typhimurium from the MLN of *P. yoelii*-infected mice, including increased dissemination to the lymph node and increased bacterial growth within the MLN. One factor promoting increased bacterial dissemination to the draining lymph node of coinfected mice could be a reduction in barrier function of the epithelium resulting from malaria parasite infection.[28] In addition, reduced killing of invasive *S.* Typhimurium in the intestinal mucosa could result from reduced neutrophil influx in the coinfected mice, as it has been shown that neutrophils are important for controlling *S.* Typhimurium in both the ileal mucosa and the MLN.[49] An increase in net bacterial replication in the MLN of *P. yoelii*-infected mice could result from reduced bacterial killing by phagocytes in the MLN, where a rare population of $CD11b^+$ $Gr1^-$ cells has been shown to harbor *S.* Typhimurium.[50] Considering the known effects of IL-10 on phagocyte microbicidal function[51,52] and the important role of neutrophils in limiting *S.* Typhimurium infection,[53] it is likely that multiple factors may contribute to the increased extraintestinal infection observed in the coinfected mice.

In conclusion, our results suggest that in malaria the immune regulation that prevents tissue-damaging inflammation and immunopathology can also limit mucosal responses to invasive bacterial infection at the level of the gut, thereby altering the clinical presentation of individuals with NTS infection. As a consequence, the diagnosis of concurrent NTS infection may not be made until antimalarial treatment has failed to improve a child's symptoms. Our results provide mechanistic insights into how, in the setting of severe malaria, mucosal responses to a bacterial pathogen are altered to effect a change in the clinical presentation of infection and broaden our understanding of how simultaneous infection with multiple pathogens can affect disease outcome.

MATERIALS AND METHODS .

Animal experiments. All animal experiments were approved by the UC Davis Institutional Animal Care and Use Committee and were performed in accordance with institutional guidelines on animal welfare.

Rhesus macaques. Four healthy, male rhesus macaques age ranging from 2–4 years, and free from *Salmonella*, *Shigella*, and *Campylobacter* (MK11-14) were selected for ligated ileal loop surgery. Macaques were inoculated intravenously with 1×10^6–2×10^6 *P. fragile*-infected rhesus macaque erythrocytes (obtained from JoAnn Sullivan at CDC). To prevent fatal infection, animals were treated with 150 mg quinine sulfate (Qualaquin; URL Pharma, Philadelphia, PA) via orogastric intubation for 2 consecutive days when parasitemia rose above 0.5%. This led to a chronic parasitemia. On day 14 after malaria parasite infection, ligated ileal loop surgery was performed, as described previously.[17] Macaques were pre-anesthetized with ketamine (10 mg kg^{-1}; Parke-Davis), followed by placement of an endotracheal tube and maintenance of the anesthesia with isofluorane. When needed, macaques were kept under a positive-pressure respirator. A laparotomy was performed, exposing the ileum and ligating 13 loops with an average of 4 cm in length, leaving 1-cm spacer loops in between. Loops were inoculated by intralumenal injections of 1 ml of either sterile LB or a logarithmically grown culture in LB containing 1×10^9 colony-forming units of wild-type *S.* Typhimurium (IR715). Loops were collected at 2, 5, or 8 h after inoculation. Uninfected control macaques ($n = 5$), designated MK1, MK5, MK7, MK8, and MK9, were from a previous study,[17] in which the ligated ileal loop surgery was performed on macaques from the same colony in an identical manner by the same surgeon (RLS).

Mice. Specific pathogen-free 6-8 week-old female CBA/J, C57BL/6J, and C57BL/6J $IL10^{-/-}$ (B6.129P2-*Il10*^{tm1Cgn}/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in specific pathogen-free caging conditions by the UC Davis Center for Laboratory Animal Science. $IL10^{-/-}$ mice were evaluated before use for signs of inflammatory bowel disease, and mice with abnormal fecal pellets or weight lower than the normal range were excluded from the study.

Plasmodium yoelii nigeriensis (P. yoelii). Parasite stocks were obtained from the Malaria Research and Reference Reagent Resource, and the species and strain identities were confirmed by DNA sequencing of merozoite surface protein-1.[19] Parasite stocks were made by passage in CD-1 mice. For coinfection experiments, mice were inoculated intraperitoneally on day 0 with approximately 4×10^7 infected RBCs in 0.1 ml of saline. Mock-infected controls were injected with the same amount of blood from CD-1 mice.

***Salmonella enterica* serotype Typhimurium**. *S.* Typhimurium strain IR715(pHP45Ω), resistant to nalidixic acid, ampicillin, and streptomycin, was used for this study.[54,55] Mice received 20 mg of streptomycin (Sigma, St Louis, MO) intragastrically 24 h before infection.[20] *S.* Typhimurium strain D23580, a multidrug-resistant bloodstream isolate from a Malawian child with malaria and NTS bacteremia,[7] was obtained from R Heyderman. Mice were inoculated with either 0.1 ml of sterile Luria-Bertani (LB) broth or 1×10^8 colony-forming units of *Salmonella* in 0.1 ml of LB broth by gastric gavage. Inocula were cultured for 16 h aerobically with selective pressure (50 mg l^{-1} carbenicillin) at 37 °C.

Histopathology. Histological samples were collected at the time of necropsy. In all, 5-μm sections were cut from formalin-fixed, paraffin-embedded tissues and stained with hematoxylin and eosin. Two trained pathologists (BPB and MNX) performed histopathology scoring in a blinded fashion, according to the scoring criteria reported previously.[48]

RNA extraction, reverse transcription-PCR, and real-time PCR. Animal tissues were frozen in liquid nitrogen at the time of necropsy and stored at −80 °C. RNA was extracted from tissue as described previously[56] using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the instructions of the manufacturer. All RNA was treated with DNAseI (Ambion, Grand Island, NY) to remove genomic DNA contamination. For a quantitative analysis of mRNA

levels, 1 μg of total RNA from each sample was reverse transcribed in a 50-μl volume (TaqMan reverse transcription reagent; Applied Biosystems, Grand Island, NY), and 4 μl of cDNA was used for each real-time reaction. Reverse transcription-PCR was performed using the primers listed in **Supplementary Table S2**, SYBR green (Applied Biosystems), and ViiA 7 real time PCR system (Applied Biosystems). Data were analyzed by using the comparative threshold cycle (C_T) method (Applied Biosystems). For macaques, target gene transcription for each sample was normalized to the respective levels of ACTB mRNA and represented as fold change over gene expression in mock-infected, adjacent loop. For mice, target gene transcription for each sample was normalized to the respective levels of *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) mRNA and represented as fold change over gene expression in control animals.

***In vivo* IL-10 blockade**. CBA mice were treated intraperitoneally with rat anti-mouse IL-10 immunoglobulin G1 (IgG1) kappa (eBioscience, San Diego, CA) on days 7 (100 μg), 9 (200 μg), and 11 (200 μg) after *P. yoelii* infection. Control mice received the same amounts of rat IgG1 kappa (BD Pharmingen, San Diego, CA) at the same time points.

Statistical analysis. All data were analyzed using the Kolmogorov and Smirnov test to determine normality, and when the s.ds. were equal, using the GraphPad InStat (GraphPad Software Inc., La Jolla, CA). The statistical significance of differences between groups was determined by a Student's *t*-test on normally distributed data with similar s.ds. For significant differences among groups with a non-Gaussian distribution, a Mann-Whitney *U* test was used. For comparison of multiple groups, analysis of variance with an appropriate posttest was used. A *P*-value of $\leqslant 0.05$ was considered to be significant. All data were analyzed using the two-tailed tests.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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