Concomitant Infection Decreases the Malaria Burden but Escalates Relapsing Fever Borreliosis[▽][†][‡]

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About 500 million cases of malaria occur annually. However, a substantial number of patients who actually have relapsing fever (RF) *Borrelia* infection can be misdiagnosed with malaria due to similar manifestations and geographic distributions of the two diseases. More alarmingly, a high prevalence of concomitant infections with malaria and RF *Borrelia* has been reported. Therefore, we used a mouse model to study the effects of such mixed infection. We observed a 21-fold increase in spirochete titers, whereas the numbers of parasitized erythrocytes were reduced 15-fold. This may be explained by polarization of the host immune response toward the intracellular malaria parasite, resulting in unaffected extracellular spirochetes and hosts that succumb to sepsis. Mixed infection also resulted in severe malaria anemia with low hemoglobin levels, even though the parasite counts were low. Overall, coinfected animals had a higher fatality rate and shorter time to death than those with either malaria or RF single infection. Furthermore, secondary malaria infection reactivated a quiescent RF brain infection, which is the first evidence of a clinically and biologically relevant cue for reactivation of RF *Borrelia* infection. Our study highlights the importance of investigating concomitant infections *in vivo* to elucidate the immune responses that are involved in the clinical outcome.

In the developing world, concomitant infections are the rule rather than the exception, and the complete clinical picture involves several microorganisms that influence each other as well as the host (8). Malaria is by far the most devastating acute febrile illness in humans, and it is caused by parasitic protozoa of the genus *Plasmodium*. The World Health Organization (WHO) has estimated that this disease is responsible for 1.5 to 2.7 million deaths annually, and about 1 million of those cases occur in children under the age of 5 years (24, 28). Considering that the most prominent symptom of malaria is fever (4) and that the WHO advises that a presumptive malaria diagnosis be made in areas where malaria is endemic, it is plausible that misdiagnoses can be made in patients who are not suffering from malaria or who have a concomitant infection (14). This assumption is supported by the results of a field study conducted by our research team in Togo, West Africa, in which 8.8% of febrile patients diagnosed as having malaria were subsequently found to have relapsing fever (RF) borreliosis, which is caused by spirochete bacteria of the genus *Borrelia* (21). This could be simply due to incorrect malaria diagnosis, which is plausible since both *Plasmodium* and RF *Borrelia* cause systemic infections with similar manifestations that involve recurrent fever, anemia, and hepatosplenomegaly. It could also be due to malaria/RF coinfection, which makes a correct diagnosis even more complex. Moreover, medical per-

sonnel are generally not aware of the existence of RF borreliosis, even though the incidence of that condition in countries such as Senegal is the highest described in Africa for any bacterial disease (26). It is possible that other infections, such as bacterial diarrheal illnesses, are underreported and might exceed the incidence of RF. To further complicate diagnosis, some patients may have malaria/RF coinfection, as was the case for 4.5% of the febrile patients in our investigation in Togo (21), and comparable findings have also been recorded in Ethiopia and Senegal (23, 26). Due to the similarity of clinical symptoms and common use of presumptive diagnostics, there is no doubt that misdiagnosis and inappropriate medication of RF often occur, as well as frequent concomitant infections.

Here, we present data from a new mouse model showing that malaria/RF coinfection results in rapid and unforeseen death that is preceded by severe anemia and serious pathological alterations of internal organs. We also demonstrate that a secondary malaria infection causes a high rate of reactivation of latent RF brain infection in mice and that a persistent RF infection attenuates the malaria infection.

MATERIALS AND METHODS

Mice. All experiments involving mice were approved by the Animal Ethical Review Committee in Umeå and were performed in accordance with Swedish animal welfare guidelines. Male BALB/c mice were purchased from Taconic (Ry, Denmark).

Analysis of parasite and spirochetal growth in mice. Groups of six BALB/c mice were injected intravenously with 1×10^7 blood-stage *Plasmodium berghei* NK65 malaria parasites and/or were injected subcutaneously with 1×10^5 *Borrelia duttonii* 1120K3 organisms to achieve single or concomitant infections. From each mouse infected with *P. berghei*, thin blood smears were prepared daily (starting on day 3) and stained with Giemsa reagent (Sigma). At least 1,000 erythrocytes were examined in each smear, and the percentage of parasitized erythrocytes (i.e., parasitemia) was calculated. Tail vein blood (5 μl) was collected daily in sodium citrate buffer from mice infected with *B. duttonii*, and the spirochetes were counted by phase-contrast microscopy.
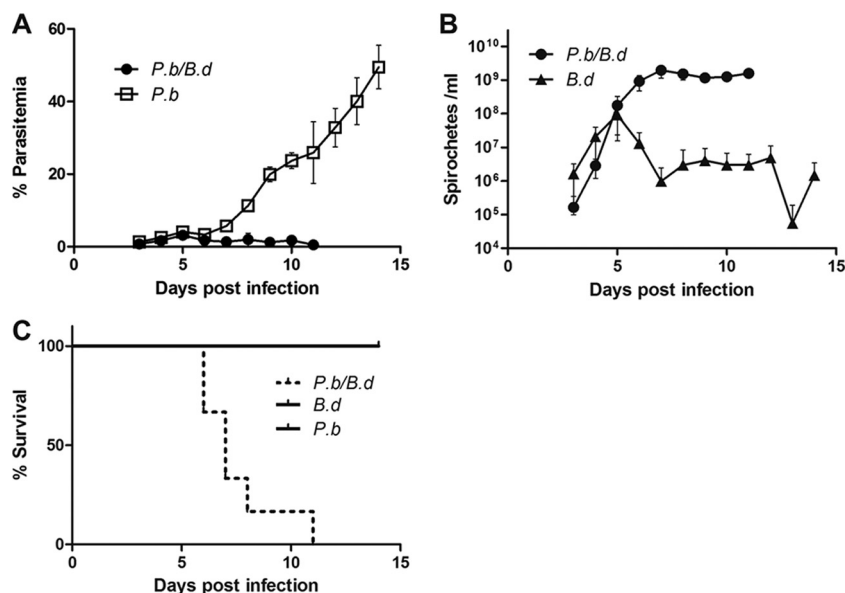
FIG. 1. Coinfection leads to decreased malaria burden, increased relapsing fever, and decreased survival. (A) Percentage of erythrocytes containing malaria parasites (designated parasitemia) in mice infected with *P. berghei* (*P.b*) or coinfected with both *P. berghei* and *B. duttonii* (*P.b/B.d*) (*n* = 6; values are means ± standard deviations [SD]). The parasitemia was significantly lower (*P* = 0.024) in coinfected mice. (B) Numbers of *B. duttonii* spirochetes in the blood of mice infected with *B. duttonii* (*B.d*) or coinfected with both *P. berghei* and *B. duttonii* (*P.b/B.d*) (*n* = 6; values are means ± SD). Spirochete titers were significantly higher (*P* = 0.014) in coinfected mice. (C) Kaplan-Meier survival curve comparing mice infected with *P. berghei* (*P.b*) with those coinfected with both *P. berghei* and *B. duttonii* (*P.b/B.d*) (*n* = 6; values are means ± SD).

Quantitative measurements of IFN-γ and IL-4 in mouse serum. Serum samples from infected mice were collected on days 0, 3, 5, 8, 11, and 14 postinfection. Cytokine concentrations were determined using a Quantakine mouse gamma interferon (IFN-γ) enzyme-linked immunosorbent assay (ELISA) and a Quantakine mouse interleukin 4 (IL-4) ELISA (R&D Systems) according to the manufacturer's instructions.

Reactivation of persistent *Borrelia* infection. Groups of 10 BALB/c mice were infected subcutaneously with 1×10^5 *B. duttonii* organisms, and 80 days later they were given an intravenous injection of 1×10^7 blood-stage *P. berghei* parasites or saline as a negative control. Spirochete and parasite burdens were determined daily as described above.

Hb measurements. Tail vein blood was collected daily, and the hemoglobin (Hb) concentration was measured using a HemoCue hemoglobin analyzer (HemoCue, Dronfield, United Kingdom).

Immunohistochemical analysis of spleen. Spleens taken from mice at 2 weeks postinfection were embedded in O.C.T. compound (Tissue-Tec; Sakura) and cut into 8-μm sections. The sections were fixed in paraformaldehyde (Sigma) and stained with anti-CD11c (Serotec) for dendritic cells, with mouse anti-B220 (Serotec) for B lymphocytes, and with mouse anti-LyG6 (BD Pharmingen) for neutrophils. The sections were subsequently treated to reduce background staining, incubated with a biotinylated rabbit anti-rat antibody followed by Strepta-ABComplex/horseradish peroxidase, and then developed with 3,3′-diaminobenzidine (Dako). Thereafter, the sections were counterstained with methyl green (Sigma) and examined in a light microscope.

Statistical analysis. Analysis of variance was done using the GraphPad Prism software package. Spirochete titers (non-Gaussian) were analyzed by the Mann-Whitney U test, and parasitemia and differences in hemoglobin levels were analyzed by Student's *t* test.

RESULTS

Concomitant infection decreases parasitemia but increases spirochete titers. Despite the high prevalence of malaria/RF coinfection in humans, there are no clinical or experimental data describing the outcome of that condition. Therefore, we assessed the outcome of concomitant malaria/RF infection in mice by monitoring growth of the rodent malaria parasite *P. berghei* and the spirochete *B. duttonii* daily from day 3 postin-

fection. In mice, *P. berghei* infection is lethal (27), whereas infection with *B. duttonii* is generally not fatal (18). In experiments in which mice were infected only with *P. berghei*, 49.5% of the erythrocytes were parasitized (here referred to as parasitemia) at day 14 postinfection (Fig. 1A). By comparison, in mice infected with both organisms, the malaria parasite load was suppressed 15-fold (*P* = 0.024), and the level of parasitemia was never more than 3.2%. (Fig. 1A). Despite the low parasitemia, the spirochete titers increased 21-fold compared to what was observed in mice infected with only *B. duttonii* (*P* = 0.014), which displayed an ordinary relapsing, self-limiting RF infection (Fig. 1B and C). The mice infected with both organisms succumbed to the coinfection before day 11 (Fig. 1C).

Polarization of the immune system toward malaria. To determine whether the host immune response preferentially targets *Plasmodia* or *Borrelia*, we monitored the T helper (TH) lymphocyte response throughout infection to ascertain whether it became polarized into being cell-mediated TH1 or humoral TH2. This was done by measuring the concentrations of the cytokines gamma interferon (IFN-γ) and interleukin 4 (IL-4). The mice infected only with malaria and those with a concomitant malaria/RF infection had similar levels of IFN-γ (Fig. 2A), and the same pattern was seen when IL-4 was measured (Fig. 2B). On the other hand, mice infected only with *B. duttonii* exhibited a TH2 cytokine pattern that was concurrent with manifestation of RF borreliosis (Fig. 2). To further analyze this immune polarization, we investigated whether the outcome of the disease might depend on which pathogen enters the host first. Mice either were infected initially with *P. berghei* and 5 days later with *B. duttonii* or were infected first with *B. duttonii* and 5 days later with *P. berghei*. Monitoring of parasitemia showed that the infections essentially followed the
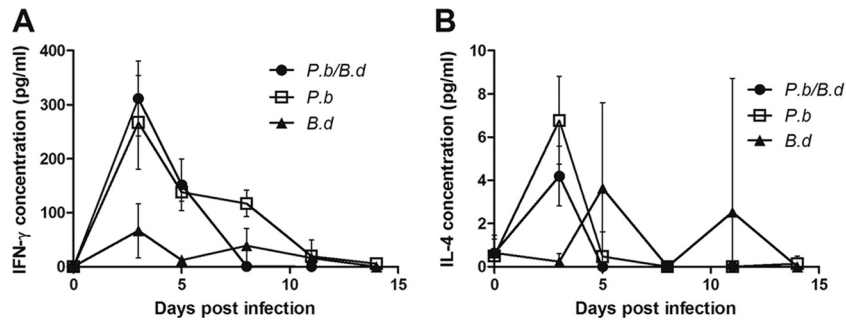
FIG. 2. The host immune response is polarized toward malaria in coinfected mice. (A) Concentrations of IFN-γ in sera from mice infected with *P. berghei* (*P.b*), with *B. duttonii* (*B.d*), or with both parasites (*P.b/B.d*) (*n* = 6; values are means ± SD). (B) Concentrations of IL-4 in sera of mice infected with *P. berghei* (*P.b*), with *B. duttonii* (*B.d*), or with both parasites (*P.b/B.d*) (*n* = 6; values are means ± SD).

pattern observed after simultaneous infection with the two pathogens (Fig. 1A; see Fig. S1A in the supplemental material). Mice receiving *P. berghei* prior to the *B. duttonii* infection had escalated spirochete titers similar to those observed earlier during simultaneous infections (Fig. 1B; see Fig. S1B in the supplemental material). A secondary *P. berghei* infection initially had a moderate effect on the *B. duttonii* infection but at later stages promoted a series of sequential relapses with spirochete titers equivalent to the first peak (see Fig. S1B in the supplemental material). Taken together, these results indicate that the immune response in concomitantly infected animals is polarized predominantly toward the malaria parasite, regardless of when the malaria infection is established.

Reactivation of latent residual RF *Borrelia*. After the febrile episodes, RF can persist in the brain as a silent, residual infection that can be activated by immune suppression (18). Therefore, we performed experiments to determine whether the polarized immune response toward malaria could reactivate residual spirochetes. Mice were infected with *B. duttonii*, and 80 days later, when the spirochetes were in their persistent and silent state, the animals were also infected with *P. berghei*. This subsequent malaria infection reactivated the spirochetes in 6 out of 10 mice (Table 1), which can be compared with reactivation in 3 out of 11 mice induced by cortisone injections (18). Moreover, the first spirochetes were seen in blood sam-

ples as early as day 13 after introduction of the malaria parasites, which was 19 days earlier than after cortisone treatment (18). This suggests that the immune response mounted against a residual *B. duttonii* infection can be disarmed by a secondary malaria infection, which is the first finding of a biologically and clinically relevant cue to reactivate latent RF *Borrelia*.

Latent residual RF infection attenuates malaria infection. As mentioned above, malaria expansion was repressed by coinfection with RF *Borrelia* (Fig. 3). To ascertain whether a latent residual RF infection would have the same effect, age-matched uninfected animals and animals with a residual *B. duttonii* infection were infected with *P. berghei*. Interestingly, the parasitemia in mice with latent residual RF was significantly lower during the first 18 days of malaria infection (*P* = 0.046) than that in age-matched control animals, suggesting that a silent residual RF infection provided some protection against malaria. However, the levels of parasitemia after day 18 postinfection were similar in the two groups of infected animals (Fig. 3).

Concomitant malaria/RF infection causes severe anemia. It has been found that mice infected with *P. berghei* develop hemolytic anemia as a result of hyperparasitemia, whereas severe malarial anemia (SMA) in humans is consistently observed to be associated with low parasite burdens (11). Therefore, we measured hemoglobin (Hb) levels in parallel with

TABLE 1. Malaria infection reactivates persistent RF *Borrelia*

Mouse	Day after malaria infection when spirochetes were detected in the blood	Highest spirochete titer (bacteria/ml)
A	13	8.6×10^8
B	18	+[a]
C	20	2.02×10^8
D	24	+
E	21	2.98×10^8
F	24	+
G	—[b]	—
H	—	—
I	—	—
J	—	—

[a] +, the spirochete titer was nonquantifiable by microscopy; the presence of spirochetes was established by concentrating the sample by centrifugation.
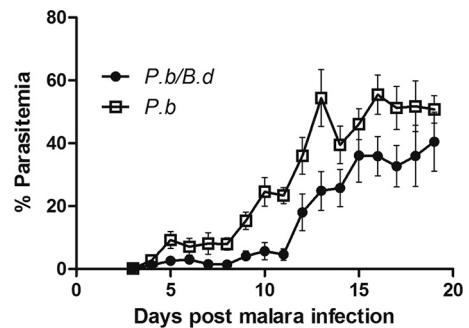[b] —, no spirochetes were detected.

FIG. 3. Latent residual RF attenuates malaria. Percentages of erythrocytes infected with malaria parasites (designated parasitemia) in mice with a residual *Borrelia* infection and a secondary malaria infection (*P.b/B.d*) or in mice with only a primary malaria infection (*P.b*) are shown (*n* = 10; values are means ± SD). The parasitemia in mice with latent residual RF was significantly lower during the first 18 days of malaria infection (*P* = 0.046) than that in age-matched control animals.
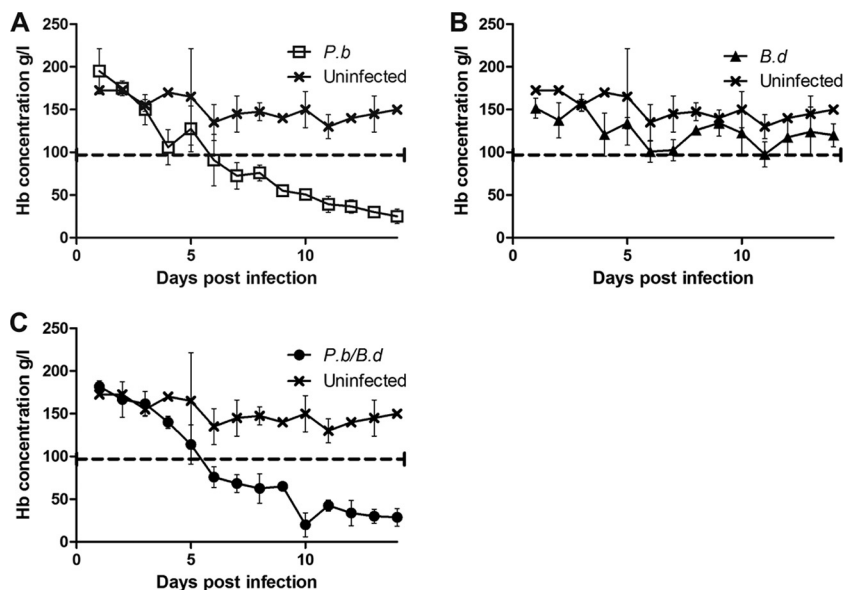
FIG. 4. *Plasmodium*/*Borrelia* coinfection leads to severe anemia. Hemoglobin (Hb) concentrations in BALB/c mice are shown. Dashed lines represent 55% of the Hb concentration determined in uninfected mice based on the definition of severe anemia in BALB/c mice (15). (A) Mice infected with *P. berghei* (*P.b*) ($n = 6$; values are means $\pm$ SD). (B) Mice infected with *B. duttonii* (*B.d*) ($n = 6$; values are means $\pm$ SD). (C) Mice infected with both pathogens (*P.b/B.d*) ($n = 6$; values are means $\pm$ SD). There was no significant difference in hemoglobin concentration between coinfected and *P. berghei*-infected mice ($P = 0.897$).

parasitemia and spirochete titers to assess the degree of anemia that arose in the animals. In BALB/c mice, *P. berghei*-induced anemia is indicated when baseline Hb levels are $\leq 55\%$ of baseline values determined in uninfected animals; by comparison, according to the WHO, SMA in humans is defined as an Hb concentration of below 50 g/liter (15). Applying these definitions, we found that RF caused only mild anemia (Fig. 4B), which confirms results reported by other investigators (13). However, both infection with *P. berghei* alone and malaria/RF coinfection led to a dramatic drop in Hb levels (Fig. 4A and C), and the mice in those experiments reached the cutoff value for severe anemia already on day 6 postinfection. This outcome was expected in the animals infected solely with *P. berghei* (Fig. 4A), but it was surprising that, similar to what is seen in human SMA, the coinfected mice developed severe anemia despite their low parasitemia ($P = 0.897$). In human malaria, this phenomenon was recently found to be enhanced by malarial suppression of the hematopoietic stem cell growth factor (22). How hematopoiesis is affected by malaria/RF coinfection is not yet known.

Concomitant malaria/RF infection severely damages internal organs. Inspecting the overall structure of the internal organs, we observed minor splenomegaly in mice infected with *B. duttonii*, whereas both the spleen and liver were significantly enlarged and darkly pigmented in malaria-infected animals. In coinfected mice, the hepatosplenomegaly was even more prominent, and several internal organs showed pathological alterations such as discolored, grayish patches, which are a sign of necrosis (Fig. 5). Therefore, we performed immunohistochemical analysis and used a blinded approach when closely assessing pathological and ultrastructural changes in the spleens of our experimental animals (Fig. 6). Spleens from mice infected with *B. duttonii* had essentially normal histolog-

ical features with intact architecture (Fig. 6) but showed an increased number of leukocytes and enlarged germinal centers. The malaria-infected animals exhibited rearranged splenic architecture (Fig. 6) (1, 10), increased hematopoietic activity, and numerous infiltrating lymphocytes, which coincided with the destruction of parasitized erythrocytes. These animals displayed no apparent Peyer's patch pathology (Fig. 5). In contrast, coinfected mice had severe necrotizing splenitis with massive infiltration of bacteria in the necrotic areas (Fig. 6) but had few phagocytes and lymphocytes. In addition, many of the lymphocytes and phagocytes were pyknotic or degenerated. Peyer's patches in the coinfected animals was enlarged and hemorrhagic (Fig. 5).

DISCUSSION

Malaria and RF occur in the same geographical areas and have been detected as coinfections in fever patients (21, 23, 26). We developed a mouse model using *P. berghei* and *B. duttonii* to study the host-pathogen interactions in simultaneous malaria/RF infection. Parasitemia was decreased 15-fold in the animals with a concomitant infection, whereas the spirochete titers were elevated 21-fold. This suggests that the rapid unexpected death of these mice was caused by uncontrollable bacteremia and possibly also SMA rather than malaria infection. Plasmodia are obligate intracellular parasites that invade and multiply within erythrocytes, but spirochetes proliferate extracellularly in the blood, and this difference has a major impact on the immune response mounted by the host. Upon infection, the TH lymphocytes play a key role in the protective adaptive immune response of the host, which can be either a cell-mediated TH1 reaction to intracellular pathogens such as malaria parasites (9, 17) or a humoral TH2 response to
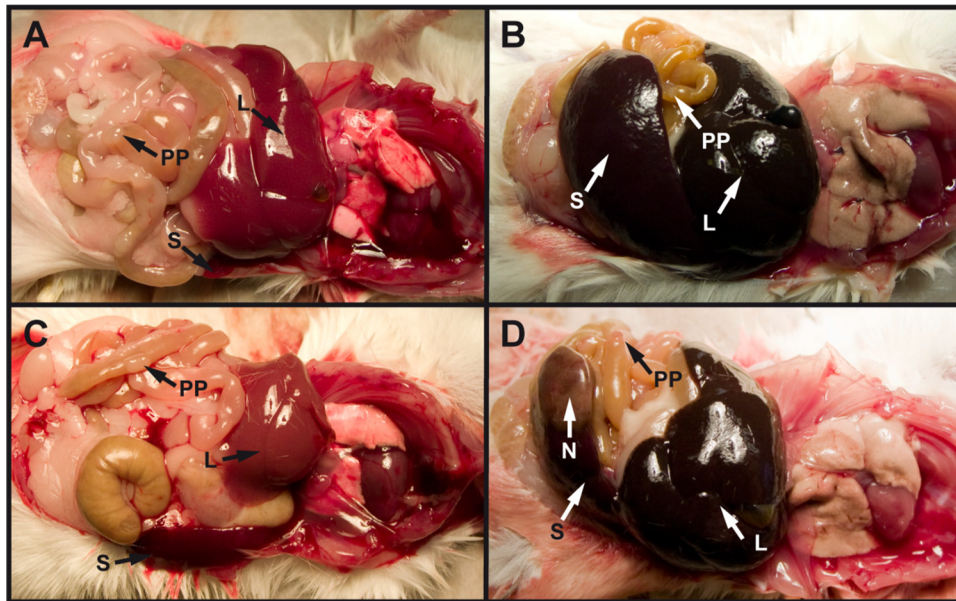
FIG. 5. Gross pathology in mice infected with both *P. berghei* and *B. duttonii*. Pictures are of internal organs from four representative BALB/c mice infected as follows: (A) uninfected; (B) infected with *P. berghei*; (C) infected with *B. duttonii*; and (D) infected with both pathogens. Abbreviations: S, spleen; N, necrotic area of the spleen; L, liver; PP, Peyer's patches. Note that the Peyer's patches in panel D are hemorrhagic.

extracellular pathogens such as RF borreliae (6, 7). TH1 cells secrete large amounts of specific cytokines, particularly IL-2 and IFN-γ, which direct the immune system to produce cytotoxic T cells and activate macrophages. The TH2 cells secrete IL-4 and additional cytokines, which induces B lymphocyte activation and subsequent antibody production (20).

There is a delicate antagonistic balance between induction of the TH1 and the TH2 responses, which depends on the signals received from antigen-presenting cells. In our study, IL-4 and IFN-γ levels were similar in mice with concomitant malaria/RF infection and those with only malaria, indicating that the TH1 response also predominates in coinfected animals. Rapidly produced IgM antibodies have been reported to serve as the first line of defense against RF *Borrelia* infection
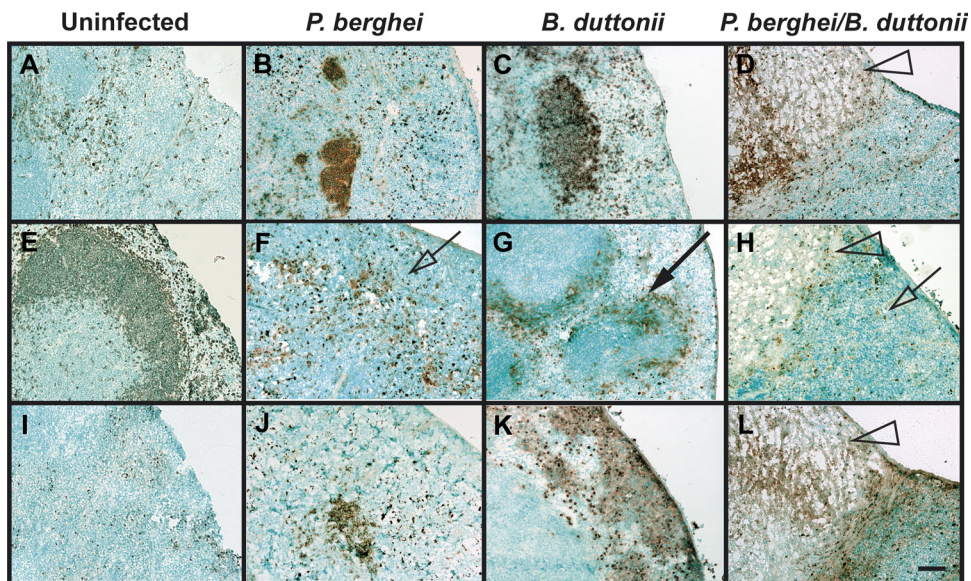

FIG. 6. Immunohistological staining of dendritic cells (A to D), B lymphocytes (E to H), and neutrophils (I to L) in murine spleen. The tissue architecture, with distinct germinal centers, is essentially normal in *Borrelia*-infected animals (arrow in panel G), while mice infected only with malaria (arrow in panel F) or with both pathogens (arrow in panel H) show a disordered structure. In the mice infected with *P. berghei* or *B. duttonii* or coinfected, immunohistochemical staining detected all three cell types, although the staining patterns could not be compared due to differences in splenic size and architecture. However, sections from the coinfected animals show severe necrotic tissue destruction (arrowheads in panels D, H, and L), which is not seen in mice infected with *P. berghei* or *B. duttonii* alone. Magnification, ×100. Scale bar, 100 μm.

(5). The seemingly uncontrolled increase in spirochetes observed in the blood of our coinfected animals might be explained by a suppressed TH2 response detected as lower IL-4 levels. In addition, most macrophages probably phagocytose malaria-infected erythrocytes, depleting the number of phagocytic cells that can keep spirochete titers down. This, together with the massive damage to the spleen, would further contribute to the rising spirochete titers associated with the fatal outcome in the coinfected mice.

It is well known that RF causes neurological symptoms such as meningitis and facial palsy (reviewed in reference 2). Latent RF brain infection has not been verified in humans. However, in mice the brain serves as an immunoprivileged site for *B. duttonii* RF, where the spirochetes are protected from the immune response outside the blood-brain barrier and can therefore persist for an extended time as a silent brain infection (18). Notably, in earlier experiments conducted by our research group (18), treatment with the corticosteroid immunosuppressant methylprednisolone was found to reactivate quiescent spirochetes, which resulted in spirochetes reentering the blood in 3 of 11 mice.

The present finding of a skewed TH1/TH2 response resulting in increased spirochete titers prompted us to address the question of whether a malaria-type immune response can promote a latent RF infection to recolonization of the blood. The faster and more efficient reactivation by malaria compared to what was observed in previous experiments using cortisone indicates that that is indeed the case, and it further strengthens the earlier suggestion of an immune-restricted but active RF infection of the brain (19, 25). Malarial immunomodulation constitutes a very plausible, epidemiologically important mechanism of residual RF reactivation, because, as mentioned above, the two diseases occur in the same geographical areas. Even more interesting is the observation that not only active but also residual RF has a protective or at least attenuating effect on secondary malaria infection. Even when RF is silent and thus not evoking an immune response in the brain, the spirochetes are metabolically active and dividing, although proliferation is most likely slow (18, 19). These spirochetes probably escape the brain at titers that are too low to cause disease but sufficient to maintain a certain immunological readiness, which in turn protects against malaria infection. Speculatively, a silent RF may even be beneficial to patients if it shields against malaria. There are cases of one disease being employed to safeguard against another. For example, before the antibiotic era, fever therapy using *Plasmodium vivax* or RF *Borrelia* was used to treat neurosyphilis (12).

Both RF and malaria cause anemia, but that condition is far milder in association with RF. The anemia in malaria is more severe due to the increasing erythrocyte lysis, which is directly correlated with the growth and maturation of the parasites. Interestingly, compared to animals infected solely with malaria, malaria/RF-coinfected animals exhibit a much more pronounced anemia, even though they show 15-fold-lower parasitemia and generally only a limited impact of RF on the anemia. The anemia that we observed during the concomitant infection in mice is very similar to the SMA seen in patients with a *Plasmodium falciparum* infection. The degree of SMA in humans is not directly correlated with the number of parasitized erythrocytes but is instead believed to be the result of additional destruction of nonparasitized erythrocytes (15), although that assumption has not yet been fully elucidated. Evans et al. used a mouse model that mimics human SMA and found evidence that uninfected erythrocytes were destroyed by a hyperactive phagocytic system (11). Therefore, in malaria/RF-coinfected mice, it is possible that the large numbers of spirochetes potentiate erythrocyte destruction and thereby attenuate the malaria infection.

The spleen is a lymphoid organ that contains macrophages and dendritic cells, which are designed to clear microorganisms from the blood, and in the mouse it is also the site of the erythropoietic response to acute anemia (3, 10, 16). It is possible that in the presence of the infectious agents, both the erythropoietic and the immunological responses can result in splenomegaly. Pathological examination of our concomitantly infected animals revealed that their spleens were enlarged and misshaped and exhibited necrotizing splenitis with massive infiltration of bacteria in the necrotic areas. The numbers of infiltrating macrophages and lymphocytes were reduced, and the majority of those cells were pyknotic or had already degenerated. None of these features were seen in animals infected with only one of the microorganisms. Besides the observed splenic and liver morbidities, coinfected animals displayed substantially enlarged and bleeding Peyer's patches, which are structures in the gut that are composed of secondary lymphoid tissue; it is tempting to assume that these pathological changes are correlated to the rising spirochete titers.

To our knowledge, this is the first article to describe an animal model of a concomitant malaria/RF infection. The mice we used displayed elevated spirochete titers but low parasitemia, which unexpectedly resulted in severe anemia similar to SMA in humans. Our results imply that coinfections might contribute to the clinical outcome of SMA and perhaps also explain the internal damage and elevated mortality seen in some malaria patients. Secondary malaria reactivates latent residual RF, whereas this residual RF infection seems to have a protective effect against malaria. Our findings suggest that concurrent infections are not merely diagnostic pitfalls that require novel strategies for diagnostics and treatment but can also lead to unexpected events in host-pathogen interactions.

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