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MAJOR ARTICLE

Malaria-Filaria Coinfection in Mice Makes Malarial Disease More Severe unless Filarial Infection Achieves Patency

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Coinfections are common in natural populations, and the literature suggests that helminth coinfection readily affects how the immune system manages malaria. For example, type 1–dependent control of malaria parasitemia might be impaired by the type 2 milieu of preexisting helminth infection. Alternatively, immunomodulatory effects of helminths might affect the likelihood of malarial immunopathology. Using rodent models of lymphatic filariasis *(Litomosoides sigmodontis)* and noncerebral malaria (clone AS *Plasmodium chabaudi chabaudi*), we quantified disease severity, parasitemia, and polyclonal splenic immune responses in BALB/c mice. We found that coinfected mice, particularly those that did not have microfilaremia (Mf^-), had more severe anemia and loss of body mass than did mice with malaria alone. Even when controlling for parasitemia, malaria was most severe in Mf^- coinfected mice, and this was associated with increased interferon-γ responsiveness. Thus, in Mf^- mice, filariasis upset a delicate immunological balance in malaria infection and exacerbated malaria-induced immunopathology.

Helminth infections are prevalent throughout tropical regions where malaria is transmitted [1–5]. Interactions among infections commonly alter disease severity [6, 7], and malaria-helminth coinfection can either exacerbate [8, 9] or ameliorate [10] the severity of disease in human hosts. Various immunological mechanisms can be invoked to explain these diverse outcomes. For example, type 1 effector mechanisms that clear intracellular pathogens and type 2 effectors induced by helminths are mutually inhibitory [11, 12]. In addition, cells and molecules that down-regulate both types of responses can be induced by helminths [13]. Helminth coinfection might thus impair the mechanisms necessary to control malaria parasitemia and/or to prevent immunopathological malaria. Before antihelminthics are widely administered in malarious areas, it is critical to understand these interactions [14].

A protection-pathology balance is at the heart of our understanding of immunity to malaria. A robust immune response is necessary to control parasite replication, but too robust a response can result in severe immunopathology [15, 16]. Malaria infection might thus be particularly sensitive to the immunological effects of coinfection. In malaria caused by *Plasmodium chabaudi chabaudi* in rodents, the severity of disease is minimized by a rapid and type 1 biased [17–19] but modulated [20–23] immune response that kills parasites and yet avoids hyperinflammation. Because coinfection with helminths can reduce type 1 effector function [24–30] and/or alter systemic levels of inflammation [25, 31] in mice, we have investigated whether preexisting filariasis alters the immunological protection-pathology balance in murine malaria.

Filarial nematodes co-occur with malaria in human populations [4, 5] and can even be carried by the same individual vector [32]. An important feature of human lymphatic filariasis is that not all hosts develop micro-

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filaremia (Mf^+; patent, transmissible infection) [33]. Similarly, in immunocompetent rodents, including the natural hosts of *Litomosoides sigmodontis* [34], only some hosts become Mf^+. In the laboratory, ~50% of *L. sigmodontis*–infected BALB/c mice become Mf^+ on day ~50 after infection [35, 36]. The mechanisms that determine Mf status are not fully understood, but the down-regulated immune responses in Mf^+ individuals [37–41] made us expect that Mf^+ mice and those that do not have microfilariae circulating in blood (Mf^-) would cope with malaria infection differently. We therefore introduced *P. chabaudi chabaudi* malaria infection at day 60 of *L. sigmodontis* infection, once the Mf status had been established.

Critically, we used epidemiology-grade quantitative analytical methods to distinguish immune-mediated disease from parasite-mediated disease and specifically assessed the respective contributions of cytokines and parasitemia to malarial severity. Quantitatively relating immune responses to clinical outcome has elucidated the course of malaria [42–45], filariasis [46], ascariasis [47], and schistosomiasis [48] in humans. Helminth-malaria coinfection, however, has yet to be addressed this way. Although it is seldom used in murine immunology, robust quantitative analysis combines powerfully with controlled laboratory experiments to improve scientific understanding [49]. With such methods, we were able to explain much of the severity of disease seen in malaria-filaria coinfection.

MATERIALS AND METHODS

Parasite life cycles and infection protocols. The filarial nematode *L. sigmodontis,* a natural parasite of the cotton rat *(Sigmodon hispidus),* was maintained by cyclical passage between gerbils *(Meriones unguiculatus)* and mites *(Ornithonyssus bacoti),* as described elsewhere [50]. Stage L3 larvae taken from mites were used to inoculate mice for these experiments. The malaria parasite *P. chabaudi chabaudi* was originally isolated from thicket rats *(Thamnomys rutilans)* and was cloned by serial dilution and passage [51]. Red blood cells (RBCs) infected with clone AS parasites [52] were passaged once through C57BL/6 mice, to provide experimental inocula.

In 3 experiments, 6–8-week-old male BALB/c mice (Harlan UK) were divided into 4 treatment groups: uninfected, infected with filaria only, infected with malaria only, and coinfected. For the filarial infections, 25 L3 larvae were injected subcutaneously. Sixty days later (with >50% of the filaria-infected mice being Mf^+), 1×10^6 malaria-infected RBCs were injected intraperitoneally [52] into each mouse scheduled to receive malaria inoculation. Mice were maintained in individually ventilated cages, with a 12-h light/dark cycle, autoclaved diet 41b (Harlan UK), and water. After 78–80 days of filarial infection (18–20 days of malaria infection), mice were killed, and their spleens were removed. Adult filariae in the thoracic cavity were counted as described elsewhere [53]. This time line and the measured variables are summarized in figure 1.

Parasitemia and disease severity data. *P. chabaudi chabaudi* parasitemia was quantified as the percentage of infected RBCs in Giemsa-stained thin-blood smears, as described elsewhere [52]. The presence or absence of circulating *L. sigmodontis* microfilariae was determined by light microscopy of thick circular smears of 10 μL of tail blood obtained on days 60–80 after filarial infection. Dried smears were rinsed in water, fixed in methanol, and stained in 5% Giemsa stain for 45 min. Mice in which no microfilariae were seen on 6 smears were considered to be Mf^-.

Disease severity was quantified in terms of loss of RBC density (a measure of anemia) and loss of body mass. RBCs were counted by use of flow cytometry of tail blood, as described elsewhere [52]. Body mass was measured on a top-pan electronic balance. Changes in RBC density and body mass were then calculated in relation to the initial measurements made for each mouse. Zeroes thus represent no change, positive values represent gains in RBC density or body mass, and negative values represent loss. Starting from day 60 (figure 1), disease severity was sampled every second day (at the same time each day), except for daily sampling during peak malaria parasitemia in 1 experiment.

Such data can be formulated in several different but strongly correlated ways: maximal, repeated, and cumulative measures of malaria parasitemia and disease severity [52]. The results below focus on the maximum percentage of RBCs parasitized (peak malaria parasitemia) and the area under the severity-versus-time curve (cumulative disease severity). Disease severity data thus capture cumulative effects of infection and are expressed in 1×10^9 RBC-days/mL (for anemia) and gram-days (for body mass). Analyses of peak disease severity, cumulative parasitemia, and peak or cumulative parasite density (per milliliter of blood) all yielded conclusions identical to those presented below.

Cytokine data. Splenic production of interferon (IFN)–γ and interleukin (IL)–4, the signature cytokines of type 1 and 2 immune responses, respectively, was quantified by ELISPOT. Briefly, ELISPOT plates (Millipore) were coated with 750 ng/well of anti–IL-4 11B11 or anti–IFN-γ R4-6A2 (both from Pharmingen) and incubated at 4°C overnight. Plates were washed with Tris-buffered saline with 0.5% Tween-20 (TBST), blocked with 2% milk at 37°C, and washed again. After RBC lysis, splenocytes were suspended in Dulbecco's modified Eagle medium (Sigma) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine (all from Gibco), and 0.5% mouse serum (Sigma). Splenocytes were plated at concentrations of 5×10^5/well and cultured in duplicate with media alone or with 1 μg/mL concanavalin A (ConA) added. Plates

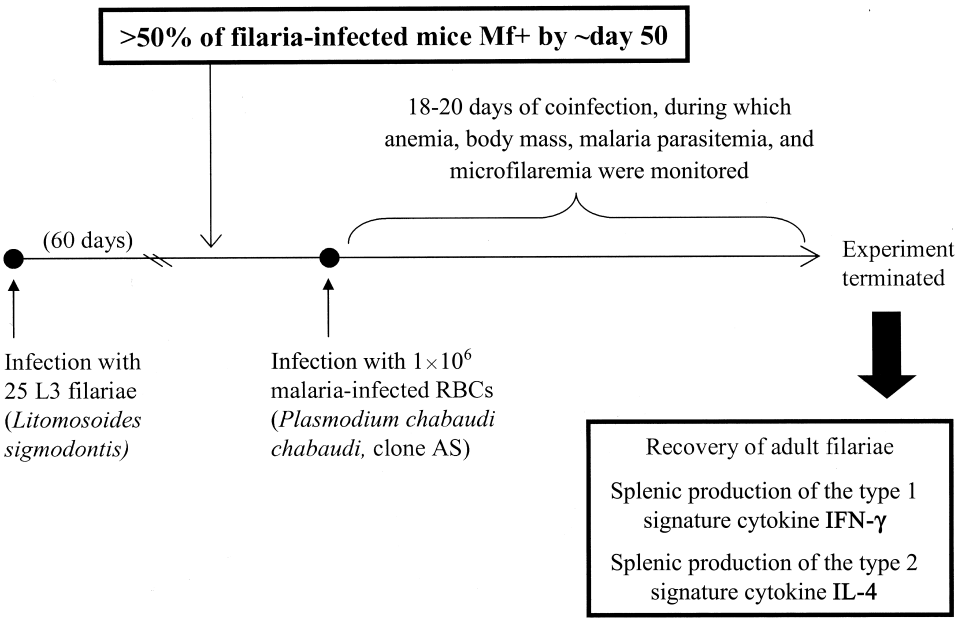

Figure 1. Time line for malaria-filaria coinfection in BALB/c mice and summary of variables measured. Three replicate experiments were performed, and data on a total of 102 mice were analyzed. These included 14 uninfected, 37 filaria-infected (27 that had microfilaremia [Mf^+]), 13 malaria-infected, and 38 coinfected (21 Mf^+) mice. IFN, interferon; IL, interleukin; RBC, red blood cell.

were incubated at 37°C (in 5% CO_2) for 72 h. After TBST and ddH_2O washes, biotinylated antibody was added (for IFN-γ, 50 ng/well of clone XMG1.2; for IL-4, 5 ng/well of clone BVD6–24G2; both from Pharmingen). Plates were incubated at 37°C for 2 h and washed with TBST, and then 50 μL/well of Extravidin AP (Sigma), diluted to 1:25,000, was added, and the plates were incubated for 30 min. After TBST and ddH_2O washes, splenocytes were stained with 100 μL/well of bromochloroindolyl phosphate/nitroblue tetrazolium substrate (Moss).

Developed plates were photographed by ImmunoSpot (CTL). Spots (i.e., cytokine-producing cells) were counted and measured by use of ImmunoSpot software (version 2.08; CTL). The number of cytokine-producing cells and the total number of cytokines (in square millimeters per well) were highly correlated (data not shown).

Statistical analyses. Analyses were conducted in SAS (version 8), by use of mixed, logistic, or general linear models [54]. Analyses focused on 102 mice: 14 uninfected, 37 filaria infected (including 27 Mf^+), 13 malaria infected, and 38 coinfected (21 Mf^+). Data from duplicate ELISPOT wells were averaged, and the number of cells producing cytokines in medium was subtracted from the number responding to ConA, before $\log_{10}(n+1)$ transformation. Counts of adult filariae were square-root transformed. No other transformations were necessary.

Qualitative differences due to infection and Mf status were consistent across experiments, but quantitative differences were strong. In other words, all experiments yielded the same conclusions, despite variations in the mean number of, for example, IL-4–producing cells or RBCs observed. Inclusion of experimental block as a factor in all analyses, plus the inclusion of initial body mass as a covariate (to account for differences among mice in initial conditions [55]), controlled for these confounding factors. (They were removed from the model whenever they were insignificant.) Experiment and infection were fitted as fixed factors, and their interaction was tested for significance. Mf status was fitted within infection. Maximal models (including interactions) were always assessed, but covariates were best fitted with common-slopes models [56]. Quadratic terms were never significant. Reported parameter estimates, including slopes (for continuous variables) and least-squares mean estimates of differences (for categorical variables), were taken from the relevant minimal model (which included significant terms only). The cutoff for significance was $P < .05$, but this was adjusted by use of Tukey's test when necessary.

Disease severity data were analyzed in 3 rounds. The first round tested for infection-related differences in anemia and loss of body mass across all treatment groups. The specific hypothesis that the severity of coinfection was an additive function of malarial plus filarial severity was also tested. The next round focused on mice infected with malaria, with a peak parasitemia covariate added to the first-round model. This addressed whether mice infected with malaria only, Mf^+ coinfected mice, and Mf^- coinfected mice had differential disease severity for a given malaria burden. The final round explored whether cytokine responsiveness was further predictive of severity of malaria in Mf^+ or Mf^- coinfected mice. Peak malaria parasi-

temia, IFN-γ–producing cells, IL-4–producing cells, and adult filarial counts were included as predictor variables. Minimal models were confirmed via backward and forward stepwise regression methods.

RESULTS

Severe disease in coinfected mice. To assess the effect of preexisting filariasis on the severity of malaria, we measured RBC density and body mass of uninfected, filaria-infected, malaria-infected, and coinfected mice. Across all experiments, coinfected mice had more severe disease, in terms of anemia (figure 2*A*) and loss of body mass (figure 2*B*) than did mice infected with malaria only ($P < .05$). These differences are discussed below. Filarial infection was avirulent as measured by its effect on RBC density and body mass ($P \sim .95$ and $P \sim .40$, vs. uninfected mice).

Severe disease in coinfected mice partially independent of parasitemia. The percentage of RBCs infected with malaria was analyzed in relation to disease severity. The exact extent of anemia and loss of body mass differed significantly in the experiments, but all 3 experiments showed that a high level of parasitemia was associated with low RBC density and low body mass (table 1). However, for a given parasitemia level, coinfected mice had more severe disease than did mice infected with malaria only (figure 3*A* and 3*B* and table 1). The difference in the severity of anemia ($6.03 \pm 2.66 \times 10^9$ RBC-days/mL)

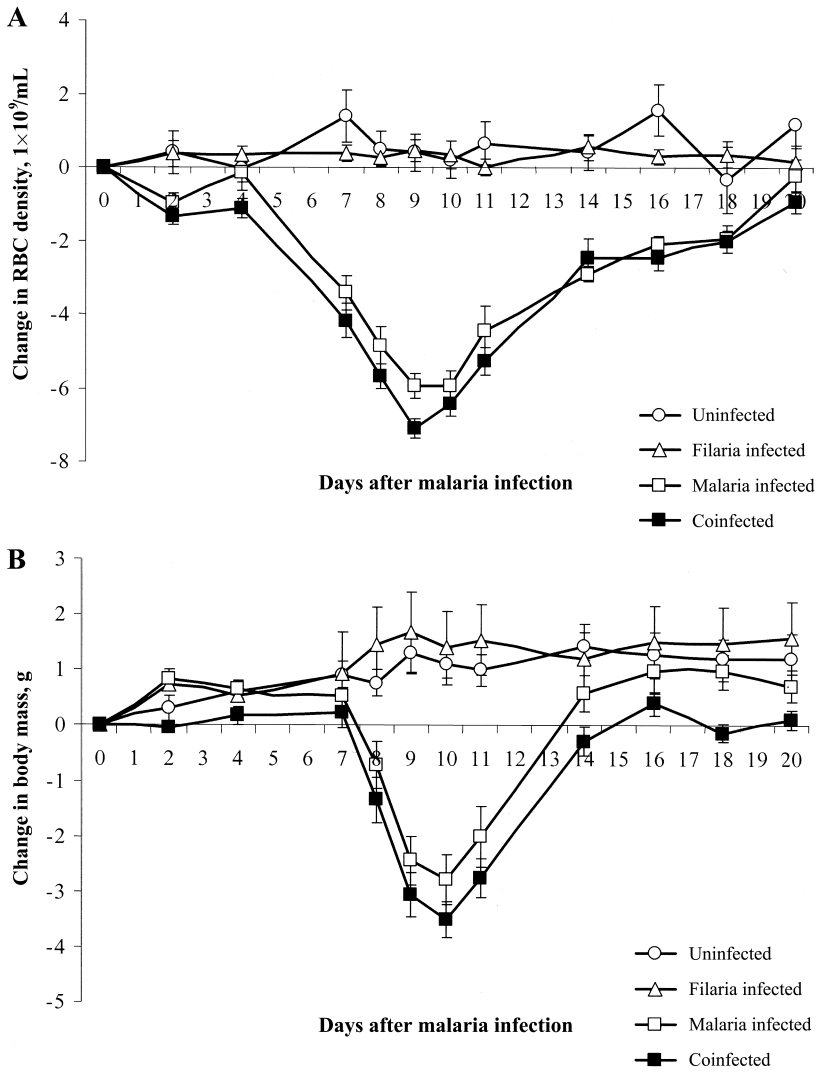

Figure 2. Anemia and loss of body mass in coinfected mice. Representative data are shown from 1 of 3 experiments of malaria-filaria coinfection (the same pattern was observed in all experiments). The initial red blood cell (RBC) density or body mass for a given mouse was subtracted from each observation, so the data are presented in terms of change from initial condition. Symbols indicate means (± SE), which were compared by use of analysis of variance. Filariasis by these measures was avirulent—filaria-infected mice were indistinguishable from uninfected mice. Coinfected mice lost more RBC density ($P < .05$; *A*) and body mass ($P < .05$; *B*) than did mice with malaria.

Table 1. For a given malaria parasitemia, coinfected mice, particularly those that did not have microfilaremia (Mf^-), had severe malaria.

Source	*df*	*F* ratio	*P* > *F*	Slope or LS mean difference (SE)
Anemia (R^2 = .63)				
Experiment	2, 47	39.53	<.0001	
(Initial body mass)	(1, 46)	(2.57)	(~.12)	
Peak parasitemia	1, 47	7.47	<.01	−0.48 (0.17)
Infection	1, 47	5.15	<.05	−6.03 (2.66)
(Microfilaremia)	(1, 45)	(0.71)	(~.40)	
Loss of body mass (R^2 = .66)				
Experiment	2, 45	7.24	<.005	
Initial body mass	1, 45	8.21	<.01	−1.56 (0.54)
Peak parasitemia	1, 45	27.74	<.0001	−0.60 (0.11)
Infection	1, 45	7.63	<.01	
Microfilaremia	1, 45	10.02	<.005	−7.55 (1.91)
				−5.89 (1.86)

NOTE. Comparison of the severity of malaria during single-species infection vs. coinfection with filaria, summarizing results for cumulative anemia and cumulative loss of body mass. The least-squares (LS) mean difference represents the difference between groups of mice after effects of experiment and the covariates (initial body mass and peak parasitemia) were taken into account. Microfilaremia was nested within infection status. Both slopes and LS mean estimates are derived from the minimal model, which excluded nonsignificant terms. The level at which nonsignificant terms were dropped from the model is shown in parentheses. Mf^+, had microfilaremia; RBC, red blood cell.

translates to a ~25% reduction in RBC density in coinfected mice (95% confidence interval [CI], 3%–46%). Mf^- coinfected mice lost more body mass than did Mf^+ coinfected (5.89 ± 1.86 g-days) and mice infected with malaria only (7.55 ± 1.91 g-days). This translates to a ~106% more severe loss of mass in Mf^-, compared with mice infected with malaria only (95% CI, 54%–159%). Neither the number of adult filariae nor any quadratic effects of malaria parasitemia significantly predicted the severity of disease in coinfected mice.

These results suggest that coinfection, particularly in Mf^- mice, altered disease severity partially independently of parasite burden. In addition, parasitemia was marginally higher among Mf^- coinfected mice (28.5 ± 1.7%) than among Mf^+ coinfected mice (23.8 ± 1.4%) ($F_{1,33} = 4.14$; $P = .05$), although levels did not differ in mice infected with malaria only versus coinfected mice overall. Mf^- coinfection may thus have slightly impaired antimalarial effectors. Still, the striking effect remains that, for a given parasitemia, disease severity was exacerbated by coinfection.

Enhanced splenic cytokine responsiveness in coinfected mice. Malarial severity has an immunopathological component, so we measured the number of IFN-γ– and IL-4–producing cells (as an indicator of cytokine responsiveness) by use of ELISPOT. Taking precise quantitative differences among experiments into account, the cytokine responsiveness of mice with filariasis was significantly higher than in those without filariasis, regardless of whether they also had malaria (figure 4*A*). Cytokine responsiveness of coinfected mice and in those infected with filaria only was thus indistinguishable, and cytokine responsiveness in mice infected with malaria only was indistinguishable from that in uninfected mice. Given that post-peak murine malaria is characterized by splenic apoptosis [57], this finding was not surprising. Importantly for the present study, both IFN-γ responsiveness ($t_{90} = 3.53$; $P < .05$) and IL-4 responsiveness ($t_{90} = 4.61$; $P < .001$) was higher in coinfected mice than in those infected with malaria only. Cytokine responsiveness in Mf^+ versus Mf^- mice was also assessed. Mf^+ and Mf^- coinfected mice did not differ in IFN-γ responsiveness ($F_{1,34} = 0.47$; $P \sim .50$) or in IL-4 responsiveness ($F_{1,34} = 0.01$; $P \sim .93$; figure 4*B*), whereas responsiveness of both IFN-γ ($F_{1,34} = 3.89$; $P \sim .06$) and IL-4 ($F_{1,34} = 3.34$; $P \sim .07$) tended to be greater in Mf^- mice infected with filaria only than in Mf^+ mice infected with filaria only (figure 4*C*).

More severe disease with increasing IFN-γ in Mf^- coinfected mice. Finally, the variation in cytokine responsiveness was used to explore variance in the outcome of coinfection. IL-4 responsiveness was not predictive, but IFN-γ responsiveness was significantly related to disease severity—the greater the number of splenocytes producing IFN-γ, the more severe the disease in Mf^- mice, in terms of both anemia (figure 5*A*) and loss of body mass (figure 5*B*). As the minimal models indicate (table 2), experimental effects on the number of RBCs or grams of body mass lost remained apparent, but the qualitative conclusions were identical, and, overall, IFN-γ responsiveness ex-

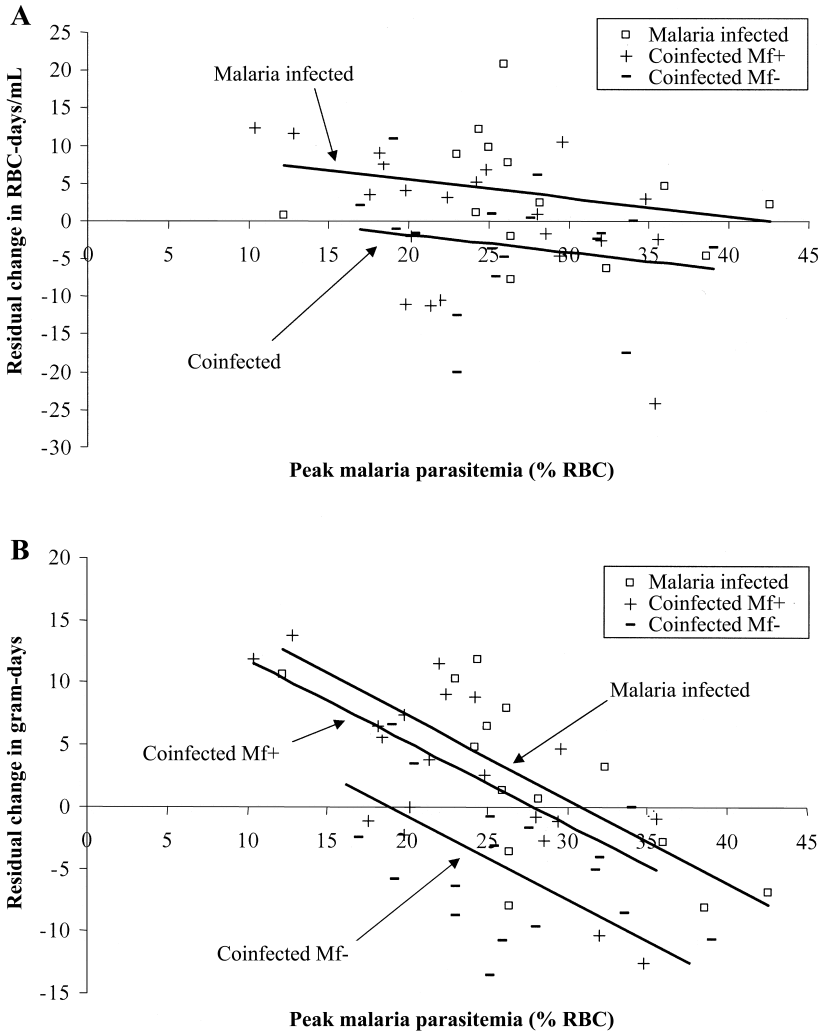

Figure 3. More severe disease in coinfected mice for a given level of malaria parasitemia. Disease severity vs. peak parasitemia for all malaria-infected mice is shown. The *y*-axes represent cumulative severity of malaria in terms of changes in red blood cell (RBC) density (anemia, in 1×10^9 RBC-days/mL, *A*) or body mass (in gram-days, *B*), after the effects of experiment and initial mass were removed. High peak parasitemia levels were associated with low RBC density and low body mass for all mice. For a given peak parasitemia level, coinfected mice had more severe disease than did mice infected with malaria only. Furthermore, coinfected mice that did not have microfilaremia (Mf^-) had significantly more severe loss of body mass than did coinfected mice that had microfilaremia (Mf^+). For detailed results of analysis of covariance, including effects of initial mass, see table 1.

plained 10% of the variance in anemia and 28% of the loss of body mass in Mf^- coinfected mice.

DISCUSSION

Malaria causes disease through parasite- and immune-mediated damage. For example, daily rounds of parasite replication lead directly to RBC lysis and anemia [58], but the severity of disease frequently exceeds the effects that are directly attributable to parasitemia [25, 59]. Immune-mediated damage is a likely cause of the decreases in temperature and body mass that are associated with malaria in mice [22, 23], and anemia itself is also partially immunopathological [15, 58]. Malaria infection thus requires a delicate immunological balance [15, 16] that might be readily upset by helminth coinfection. Indeed, we found that coinfected mice were more anemic (figure 2*A*) and lost more body mass (figure 2*B*) than mice infected with malaria only. Filariasis itself did not cause anemia or loss of body mass, yet malaria-filaria coinfection resulted in severe emergent disease. How? On the one hand, helminth coinfection can impair type 1 effector mechanisms, such that the host loses control of pathogen replication [24–30]. On the other hand, immunomodulatory effects of helminths [13] might prevent immunopathology: the best response to infection is often predominantly antiinflammatory, even if it impairs parasite killing [12]. Dis-

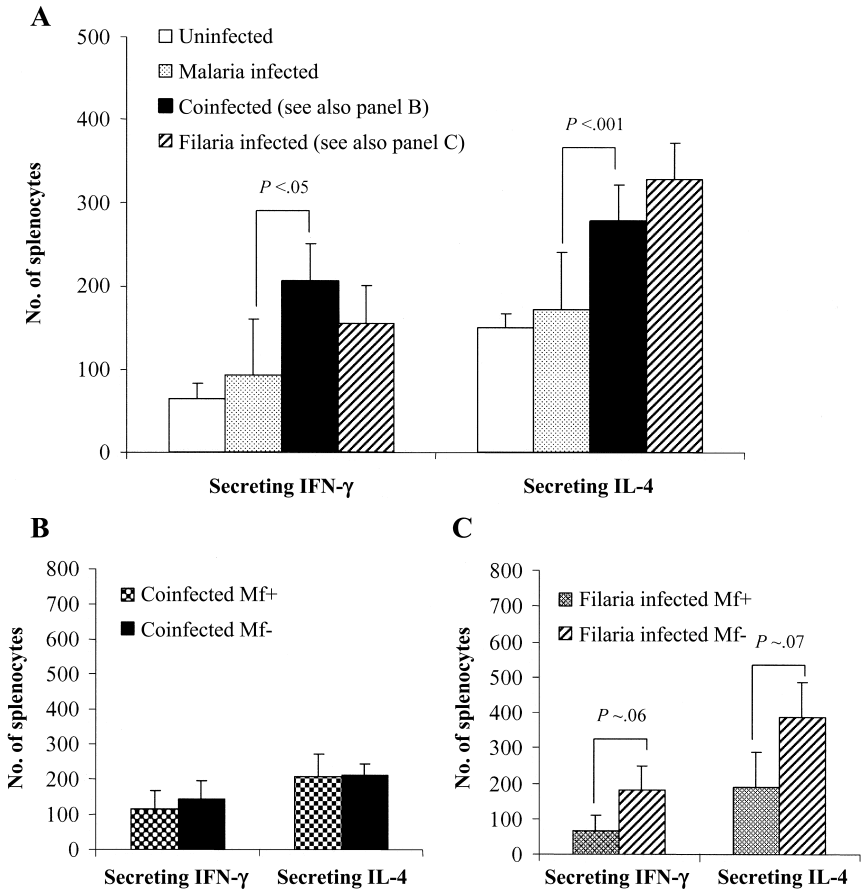

Figure 4. Enhancement of splenic cytokine responsiveness in coinfection, compared with infection with malaria alone. The no. of cells per 500,000 splenocytes producing interferon (IFN)–γ or interleukin (IL)–4 is shown. The no. of cells that made cytokine in media alone was subtracted from the no. that made cytokines in response to in vitro stimulation with concanavalin A. After log transformation, the data were subjected to analysis of variance, to remove experimental effects. The data shown were back-transformed and represent the mean (± SE) no. of cells producing each cytokine. *A,* Significantly higher production of cytokines in coinfected vs. malaria-infected mice, for both IFN-γ ($P < .05$) and IL-4 ($P < .001$). Cytokine responses of uninfected mice were not distinguishable from those of malaria-infected mice, and cytokines of filaria-infected mice were similar to those of coinfected mice. *B,* No relationship of cytokine responses to microfilaremia (Mf) status in coinfected mice ($P \sim .50$ for IFN-γ; $P \sim .93$ for IL-4). *C,* Tendency of greater mean cytokine responsiveness in Mf^- than in Mf^+ filaria-infected mice ($P \sim .06$ for IFN-γ; $P \sim .07$ for IL-4).

tinguishing the pathogenic effects of immune exuberance from the pathogenic effects of parasite replication is a major challenge, especially when they lead to the same symptoms (e.g., anemia) [58]. By sequentially adding parasitemia and IFN-γ production to statistical models, we were able to demonstrate that filarial exacerbation of malarial disease was not entirely driven by parasite levels.

Malaria parasites do damage their host directly, so it is no surprise that disease severity increases with increasing parasitemia, for both natural [60, 61] and experimental (figure 3) [52, 61] cases of malaria. For a given parasitemia, however, we found that coinfected mice had more severe anemia than did mice infected with malaria only (figure 3*A*) and that Mf^- coinfected mice lost the most body mass of all groups (figure 3*B*). A considerable proportion of this difference in severity of disease was explained by splenic IFN-γ responsiveness: both anemia and loss of body mass of Mf^- coinfected mice were more severe as the number of IFN-γ–producing cells increased (figure 5). We propose that the high IFN-γ responsiveness of these mice was symptomatic of systemic, pathological inflammation. Serum cytokines and antibody isotypes measured in a subset of the mice supports the hypothesis that their inflammation was systemic (data not shown). Furthermore, the proposed link between high IFN-γ responsiveness and immunopathological malaria is in keeping with the well-documented, negative pathological effect of excessive or late production of proinflammatory cytokines in murine malaria [21–23, 62]. The present results thus provide quantitative (albeit nonmechanistic) support for the idea that the immune system, to fight malaria optimally, must strike a delicate balance—to kill parasites yet modulate the pathological overproduction of inflammatory cytokines.

Malaria did not become more severe because of filarial co-

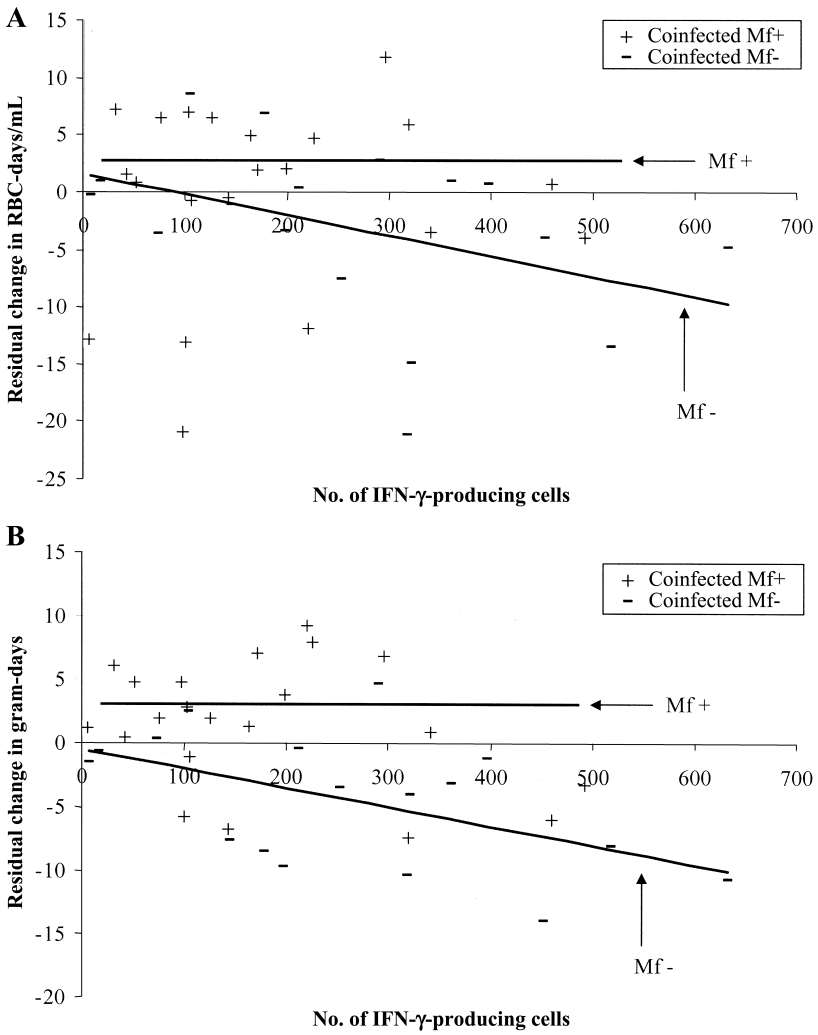

Figure 5. More severe loss of body mass with increasing production of interferon (IFN)–γ in coinfected mice that did not have microfilaremia (Mf^-). Residuals of disease severity versus back-transformed IFN-γ–producing cells, for all malaria-infected mice. The *y*-axes represent cumulative severity of malaria in terms of changes in red blood cell (RBC) density (anemia, in 1×10^9 RBC-days/mL) or body mass (in gram-days) after effects of experiment, initial body mass, and peak parasitemia were removed. High IFN-γ responsiveness was associated with low RBC density *(A)* and low body mass *(B)* in coinfected Mf^- mice. IFN-γ production did not explain variance in severity of disease in mice that had microfilaremia (Mf^+; see table 2 for detailed results of analysis of covariance).

infection per se but because of coinfection without microfilaremia. Were Mf^+ mice, simultaneously equipped with inflammatory and down-regulatory immune machinery in the blood, better prepared to cope with malaria? Conversely, were Mf^- mice made prone to severe disease by their combination of type 2 bias and insufficient immunomodulation? This would be consistent with known immunological differences between Mf^+ and Mf^- individuals. For example, Mf^+ people down-regulate immune responses, often via IL-10, such that overall responsiveness is markedly [37–40] and persistently [63] suppressed. Importantly for coinfection, Mf^+ people have suppressed responses to bystander antigens [64, 65]. For *L. sigmodontis*–infected mice, cytokine data have not yet been broken down by Mf status in the literature. What is clear, however, is that Mf in mice is controlled by IL-4 and IL-13: depletion or knockout of these cytokines [66, 67] or their common receptor ($IL\text{-}4R^{-/-}$ BALB/c mice; data not shown) increases microfilariae levels ⩾10-fold, compared with levels in wild-type. These results are paralleled by evidence that *Brugia malayi* microfilariae injected into mice are cleared by type 2 [68, 69], not type 1 [70], effector mechanisms. Of further interest, injected Mf induce IFN-γ production on their own [71, 72]. Mf^+ filariasis in mice thus entails increased IFN-γ and low IL-4 levels, compared with levels in Mf^- mice. At the same time, IL-10 is required for the long-term survival of *L. sigmodontis* microfilariae [41]. The idea that Mf^+ *L. sigmodontis* filariasis is down regulatory is also consistent with the results shown in figure 4*C*. In any case, malaria par-

Table 2. Disease became more severe with increasing interferon (IFN)–γ responsiveness in coinfected mice that did not have microfilaremia (Mf^-).

Source, by Mf status	*df*	*F*	*P* > *F*	Slope (SE)
Anemia				
Mf^+ (R^2 = .54)				
(Experiment)	(2, 17)	(0.26)	(~.77)	
Initial body mass	1, 19	22.15	<.0005	−4.56 (0.97)
(Peak parasitemia)	(1, 18)	(0.01)	(~.94)	
(Log_{10} IFN-γ cells)	(1, 18)	(0.06)	(~.81)	
Mf^- (R^2 = .77)				
Experiment	2, 13	14.27	<.0005	
(Initial body mass)	(1, 12)	(0.04)	(~.85)	
(Peak parasitemia)	(1, 12)	(0.06)	(~.81)	
Log_{10} IFN-γ cells	1, 13	5.53	<.05	−16.79 (7.14)
Loss of body mass				
Mf^+ (R^2 = .66)				
Experiment	2, 17	5.59	<.05	
(Initial body mass)	(1, 16)	(0.24)	(~.63)	
Peak parasitemia	1, 17	16.17	<.001	−0.81 (0.20)
(Log_{10} IFN-γ cells)	(1, 16)	(0.08)	(~.79)	
Mf^- (R^2 = .75)				
Experiment	2, 13	19.23	<.0005	
(Initial body mass)	(1, 12)	(3.67)	(~.08)	
(Peak parasitemia)	(1, 12)	(0.01)	(~.94)	
Log_{10} no. of IFN-γ–producing cells	1, 13	14.51	<.005	−16.55 (4.35)

NOTE. Comparison of the severity of malaria in coinfected mice that had circulating microfilariae (Mf^+) vs. coinfected mice that did not have circulating microfilariae (Mf^-), summarizing results for cumulative severity of anemia and cumulative loss of body mass. For Mf^+ mice, only initial body mass was predictive of anemia, and experiment and peak parasitemia were predictive of loss of body mass. In Mf^- mice, IFN-γ was a significant predictor of both anemia and loss of body mass, which suggests that Mf^- coinfected mice are prone to immunopathological malaria. The level at which nonsignificant terms were dropped from the model is shown in parentheses. Slopes were determined as in table 1.

asites entering mice with preexisting Mf^+ versus Mf^- filariasis were entering rather different immune environments.

The cytokines that determine Mf status could indeed have affected how well coinfected mice struck the necessary compromise between controlling parasitemia and minimizing immunopathology. In Mf^- mice, the type 2–associated cytokines that keep microfilariae at bay would tend to impair [73] or delay [74] the control of malaria, whereas IFN-γ in Mf^+ mice would promote the clearance of malaria [17–19]. This difference in the balance of type 1 cytokines could explain the lower levels of malaria parasitemia in Mf^+ mice, compared with those in Mf^- coinfected mice (23.8% vs. 28.5%). Then, down-regulatory cytokines in Mf^+ mice could have prevented the strikingly more severe disease seen in Mf^- mice—cytokines such as IL-10 [22, 23] and transforming growth factor–β [21, 23] are critical for preventing malarial immunopathology, particularly after parasitemia is controlled. In other words, the Mf^- coinfected mice may have had dual factors increasing the severity of their disease, first effector impairment and then the absence of immunomodulation. IFN-γ and IL-4 are probably not the only important factors in this system; we will next use multiplex assays to identify the role of additional cytokines in determining the severity of malaria during filarial coinfection. Another future direction is to assess the effect of the timing of coinfection. For example, would introducing malaria before day 50 of *L. sigmodontis* infection (when all mice are Mf^-) make the outcome of coinfection uniformly severe? Would the introduction of malaria after day 90 of infection (when most filariae are cleared) ablate the effects of preexisting filariasis on malarial severity? Alternatively, if malaria came first, would the severity of malarial disease be altered by incoming filariae? Because the timing of coinfection is likely to affect clinical outcome, it deserves future study.

In other systems, preexisting antihelminth immune responses affect the management of malaria in similarly complex ways. For example, pretreatment with irradiated *Brugia pahangi* larvae ameliorated cerebral malaria (and was thus to the net benefit of the mouse), even though anemia was exacerbated [25]. If antiinflammatory properties of helminths [13] can prevent cerebral malaria or other immunopathological symptoms, then the excess anemia associated with effector impairment may be a price worth paying. Different helminth-malaria combinations, however, appear to result in diverse outcomes [25, 26, 29, 75, 76], depending on, for example, the type of mouse

studied [29]. With further work in diverse systems, generalizations may begin to emerge, and the relevance of animal models of coinfection to field studies may become clear.

In any case, steps toward understanding coinfection outcomes are urgently needed. In this regard, field studies on the clinical consequences of helminth-malaria coinfection in people [2, 6, 8–10] would do well to take the lead from recent multivariate analyses of malaria immunoepidemiology [42–45]. Several of these previous studies have suggested that ratios of pro- to antiinflammatory factors predict disease severity [42, 43, 45], and one goes further to demonstrate that quantifiable relationships among proinflammatory cytokines, their soluble receptors, and malaria parasitemia together determine clinical outcome [44]. Such approaches, like the statistical methods used here to distinguish parasite- from immune-mediated disease, should prove to be powerful tools for understanding the severity of malaria in helminth-infected people. If further work reveals that Mf^+ filariasis is generally protective against severe malaria, however, application of the findings will be difficult. The immune modulation induced by Mf^+ filariasis can, for example, impair the success of antimycobacterial [64] and tetanus [65] vaccinations. In a coinfected world, health workers may have to choose which disease to prevent.

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