Counter-regulatory anti-parasite cytokine responses during concurrent *Plasmodium yoelii* and intestinal helminth infections in mice

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**A B S T R A C T**

Malaria and helminth infections are two of the most prevalent parasitic diseases globally. While concomitant infection is common, mechanisms contributing to altered disease outcomes during co-infection remain poorly defined. We have previously reported exacerbation of normally non-lethal *Plasmodium yoelii* malaria in BALB/c mice chronically infected with the intestinal trematode *Echinostoma caproni*. The goal of the present studies was to determine the effect of helminth infection on IFN-γ and other key cytokines during malaria co-infection in the *P. yoelii–E. caproni* and *P. yoelii–Heligmosomoides polygyrus* model systems. Polyclonally stimulated spleen cells from both *E. caproni* and *H. polygyrus*-infected mice produced significantly lower amounts of IFN-γ during *P. yoelii* co-infection than malaria-only infected mice. Furthermore, the magnitude of IFN-γ suppression was correlated with the relative amounts of IL-4 induced by these helminths (*E. caproni* = low; *H. polygyrus* = high), but not IL-10. Concurrent malaria infection also suppressed helminth-associated IL-4 responses, indicating that immunologic counter-regulation occurs during co-infection with malaria and intestinal helminths.

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1. Introduction

Malaria and helminthiases are the two most prevalent parasitic diseases globally, with approximately 500 million people suffering from malaria (Snow et al., 2005) and an estimated 2 billion infected with helminths (de Silva et al., 2003). Since the epidemiologic distributions of these diseases overlap in many tropical areas, concomitant infection is a frequent occurrence (Mwangi et al., 2006). Studies from human populations reveal that helminth infection can have a negative effect on host response to malaria, including increased susceptibility to *Plasmodium* infection and increased severity of disease (Nacher et al., 2002; Sokhna et al., 2004; Spiegel et al., 2003; Tshikuka et al., 1996). Conversely, protective effects, such as decreased risk of cerebral malaria and lower incidence of malaria, have also been reported for those infected with helminths (Briand et al., 2005; Lyke et al., 2005; Murray et al., 1977; Nacher et al., 2000).

Rodent models of co-infection offer a tractable means of defining the underlying mechanisms, and results from various models reflect the spectrum of interactions observed in humans (Hartgers and Yazdanbakhsh, 2006). We have previously reported that BALB/c mice infected with an intestinal trematode, *Echinostoma caproni*, were susceptible to increased duration and intensity of malaria parasitemia, resulting in approximately 50% fatality, when infected with otherwise non-lethal *Plasmodium yoelii* (Noland et al., 2005). Similar exacerbation of murine malaria during helminth co-infection has been reported for *E. caproni* (referred to as *Echinostoma revolutum* in the paper) (Christensen et al., 1988), as well as for other helminths such as the blood trematode *Schistosoma mansoni* (Legesse et al., 2004; Lwin et al., 1982; Helmy et al., 1998; Yoshida et al., 2000) and the intestinal nematode *Heligmosomoides polygyrus* (Su et al., 2005).

Host immune response to blood stage malaria infection consists of an orchestrated cytokine and antibody response. IFN-γ, the hallmark Th1 cytokine, plays a central role in the anti-malaria response by limiting parasite growth through the classical activation of macrophages (Stevenson and Tam, 1993; Li et al., 2001), and promoting cytotoxic antibody production (Snapper and Paul,
per-parasitemia and death (Su et al., 2005). Co-infection of mice with *H. polygyrus* also impairs IFN-γ production during *P. yoelii* co-infection, rendering mice susceptible to hyper-parasitemia and death (Su et al., 2005).

Here, we evaluated whether chronic *E. caproni* infection in BALB/c mice similarly leads to reduced IFN-γ response during concurrent *P. yoelii* infection. We also investigated whether IFN-γ suppression in this model was associated with production of the hallmark Th2 cytokine IL-4 or the regulatory cytokine IL-10, both of which have demonstrated activity against Th1 effector function (Groux et al., 1996; Seder et al., 1992; Skapenko et al., 2004). While the humoral immune response to *E. caproni* infection in various rodent hosts has been well described (Toledo et al., 2006), the cellular cytokine response in BALB/c mice is largely unknown. Therefore, to control for the relative production of IL-4, we compared the immune response during *E. caproni*-malaria co-infection in BALB/c mice with that of mice co-infected with *H. polygyrus*, an intestinal nematode of mice that induces a vigorous IL-4 response (Urban et al., 1991).

### 2. Materials and methods

#### 2.1. Animals and parasites

For all experiments, age-matched male BALB/c mice were purchased from the National Cancer Institute (Bethesda, Maryland) and maintained in a pathogen-free micro-isolation facility in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals. Mice were infected orally with either 10–15 *E. caproni* metacercarial cysts or 200 third-stage *H. polygyrus* larvae (L3) as previously described (Noland et al., 2005; Urban et al., 1991). At three weeks post-helminth infection, groups of helmint-infected and worm-free age-matched controls were infected with 10⁵ *P. yoelii* 17– non-lethal (NL) strain parasitizing erythrocytes by intraperitoneal (i.p.) injection. Infections were monitored by Giemsa-stained blood smears prepared from tail bleeds, and parasitemia calculated from examining approximately 1000 red blood cells (RBCs).

#### 2.2. Spleen cell cultures

Spleens were harvested aseptically from worm-free, *P. yoelii*-infected, *E. caproni*-infected, *E. caproni*- *P. yoelii* co-infected, *H. polygyrus*-infected, and *H. polygyrus*- *P. yoelii* co-infected mice at days 3, 5, and 10 post-malaria infection (n = 3 per time point). Spleens were placed into wells containing DMEM (Mediatech, Herndon, VA) supplemented with 10% FBS (Sigma, St. Louis, MO), 25 mM HEPES (Gibco, Carlsbad, CA), and 100 U/ml penicillin-100 μg/ml streptomycin (Gibco) (complete medium). Spleens were crushed between the frosted ends of sterile glass slides, filtered through nylon mesh, and RBCs lysed with ACK lysis buffer (Invitrogen, Carlsbad, CA). Cells were then washed with complete medium, and viability determined by trypan blue (Gibco) exclusion. Cells were re-suspended in complete medium supplemented with 50 μM 2-mercaptoethanol (Sigma) and plated at a concentration of 5 × 10⁶ cells/ml in triplicate aliquots of 750 μl into 48-well tissue culture plates pre-coated overnight with either anti-CD3 (BD Biosciences, San Jose, CA; 1 μg in 100 μl) or PBS alone. Cells were cultured for 48 h at 37°C in a humidified CO2 incubator and, following centrifugation; supernatants were collected and stored at −80°C.

#### 2.3. Cytokine ELISAs

Levels of IFN-γ, IL-4, and IL-10 were measured from splenocyte culture supernatants by sandwich ELISA using paired capture and detection antibodies and concentration calculated against a standard curve of recombinant cytokines (BD Biosciences). Preliminary assays were carried out to ensure that responses fell within the linear range of the standard curve. Capture and detect antibodies, respectively, for each cytokine were as follows: R4-6A2 and XMG.1.2 for IFN-γ; 11B11 and BVD6-24G2 for IL-4; JES5-2A5 and SXC-1 for IL-10 (all BD Biosciences). 96-well Immulon 2 plates (Thermo Electron) were coated overnight at 4°C with 100 μl capture antibody (2 μg/ml) diluted in PBS. Wells were washed with PBS/0.05% Tween 20, then blocked for 1 h at 37°C with blocking buffer: PBS/5% non-fat dry milk for IL-4, IL-10; or PBS/1% BSA (Sigma) for IFN-γ. Following washing with PBS/0.05% Tween 20, 100 μl of recombinant cytokines standards, duplicate samples diluted 1:10 in blocking buffer, or negative samples of blocking buffer alone were added to plates, and incubated for 1–2 h at 37°C. Plates were washed again, then incubated for 1 h at 37°C with 100 μl of biotinylated detection antibody (1 μg/ml) diluted in blocking buffer, followed by an additional wash and incubation with 100 μl streptavidin-peroxidase (2 μg/ml; Sigma). After extensive washing, plates were developed with 100 μl ABTS (2, 2’-azino-di-[3-ethylbenzthiazoline-6-sulfonate]; Kirkegaard & Perry Laboratories, Gaithersburg, MD), and absorbance read at 405 nm. Net cytokine levels were calculated by subtracting the mean amount of cytokine produced spontaneously in unstimulated PBS cultures from that produced in anti-CD3 stimulated cultures. Samples from each time point were analyzed simultaneously to minimize variability.

#### 2.4. IFN-γ neutralization

Mice were given i.p. injections (1 mg/mouse in endotoxin-free PBS) of anti-IFN-γ (XMG.6, a kind gift from Dr. Tom Richie at the US Navy Medical Research Center), rat IgG as control, or PBS alone at days 4, 6, and 8 post-*P. yoelii* infection. For measurement of IFN-γ in these mice during malaria infection, blood was drawn by tail bleeds at days 5 and 10 post-*P. yoelii* infection and allowed to clot over-night at 4°C. Serum was then collected and stored at −80°C, and levels of IFN-γ analyzed by ELISA exactly as described above for splenocyte cultures.

#### 2.5. Statistical analysis

Differences in cytokine levels between groups were compared using the non-parametric Wilcoxon rank-sum (Mann–Whitney) test. Association between IL-4 production and IFN-γ production in helminth-infected mice was calculated using Spearman’s rank correlation. All analyses were performed using Stata 9.2 (StataCorp, College Station, TX), and a P value <0.05 was considered significant. Results from cytokine characterization are reflective of two independent experiments.

### 3. Results

#### 3.1. IFN-γ kinetics during *P. yoelii* infection

To first determine the kinetics of IFN-γ production in BALB/c mice infected only with non-lethal *P. yoelii*, we characterized cytokine production from spleen cells over the course of malaria infection in response to polyclonal stimulation with anti-CD3. Production of IFN-γ peaked early in infection, around day 5, and then returned to baseline levels by day 10 ([Fig. 1](#fig1){refig}). This precipitous decline preceded peak parasitemia by several days, and is consis-
Next we determined whether IFN-γ production during this period (days 3–10 post- \textit{P. yoelii} infection), was impaired in mice chronically infected with either \textit{E. caproni} or \textit{H. polygyrus}. Beginning at day 3 post-malaria infection, levels of IFN-γ were significantly elevated in all malaria-infected mice, whether alone or in combination with chronic helminth infection, compared to naïve control mice or helminth-only infected mice (Fig. 2). By day 5, co-incident with rising parasitemia (Table 1), large amounts of IFN-γ were produced by spleen cells from all malaria-infected mice (Fig. 2). However, when compared to malaria-only infected mice, levels of IFN-γ were significantly reduced in both \textit{E. caproni–P. yoelii} and \textit{H. polygyrus–P. yoelii} co-infected mice (Fig. 2). By day 10 post-malaria infection, IFN-γ production returned to baseline levels in all groups.

### 3.3. Differential IL-4 response to helminth infection and correlation with IFN-γ suppression

The Th2 cytokine, IL-4, is a potent inhibitor of IFN-γ (Seder et al., 1992; Skapenke et al., 2004). Therefore, we evaluated whether suppression of IFN-γ during malaria co-infection was related to IL-4 production in helminth infected mice. Because it has previously been reported that \textit{E. caproni} infection induces only a moderate IL-4 response (Brunet et al., 2000), the magnitude of IL-4 production in \textit{E. caproni}-infected mice was compared with that of \textit{H. polygyrus}, a prototypic IL-4 inducing parasite (Urban et al., 1991). We found that between 4 and 5 weeks post-helminth infections (the period corresponding to the initiation of malaria infection in co-infected mice), levels of IL-4 produced by anti-CD3 stimulated spleen cells from \textit{E. caproni}-infected mice were significantly greater (\(P = 0.03\)) than levels produced by naïve uninfected control \textit{BALB/c} mice (Fig. 3). However, the IL-4 response in \textit{E. caproni}-infected mice was significantly lower than the robust IL-4 response from \textit{H. polygyrus} infected mice (\(P = 0.0002\)). Based on this categorization (\textit{H. polygyrus} = high; \textit{E. caproni} = low), there was a significant inverse correlation between IL-4 and IFN-γ in these groups of mice at day 5 of concurrent \textit{P. yoelii} infection (Spearman’s \(\rho = -0.88\); \(P = 0.02\))–i.e. \textit{H. polygyrus} co-infected mice dis-
played a higher degree of IFN-γ suppression than *E. caproni* co-infected mice.

### 3.4. IL-10 characterization during malaria and helminth infections

The regulatory cytokine IL-10 also exerts potent anti-inflammatory properties (Groux et al., 1996), and has been shown to play a critical role in malaria disease progression (Li et al., 1999). We therefore evaluated whether this cytokine was associated with helminth-induced IFN-γ suppression. Mice infected only with *H. polygyrus* produced significantly more IL-10 (8.28 ± 3.98 ng/ml, mean ± SD; *P* = 0.0002) over the course of the experiment than either mice infected with *E. caproni* alone (3.25 ± 2.80 ng/ml) or uninfected control mice (3.10 ± 1.17 ng/ml, *n* = 12 per group). This likely accounts for the elevated IL-10 observed at day 3 post-malaria infection in *H. polygyrus*-malaria co-infected mice versus those infected only with malaria (*P* < 0.05; Fig. 4). Infection with *P. yoelii* in worm-free and helminth co-infected mice induced a significant IL-10 response by day 5 post-malaria infections (Fig. 4). However, there was no significant difference in levels between these groups, suggesting that IL-10 production from spleen cells of helminth-infected mice had little effect on the suppression of IFN-γ observed in helminth–malaria co-infected mice.

### 3.5. Malaria-induced IL-4 suppression

These studies also permitted a detailed examination of the effect of concurrent malaria infection on the host immune response to helminth infection. Co-infection of *H. polygyrus*-infected mice with *P. yoelii* progressively reduced the high levels of IL-4 normally produced in *H. polygyrus* infection to near base-line amounts by day 10 of malaria infection (Fig. 5). Likewise, even the relatively low levels of IL-4 produced in response to infection only with *E. caproni* were significantly reduced by day 10 post-malaria infection in *E. caproni–* *P. yoelii* co-infected mice.

### 3.6. Effect of IFN-γ neutralization during acute malaria infection

In order to determine whether neutralization of serum IFN-γ in the *P. yoelii* system would recapitulate the exacerbation previously observed during helminth–*P. yoelii* co-infection (Noland et al., 2005), we administered neutralizing and control antibodies at three time points during early malaria infection (days 4, 6, and 8), and analyzed serum levels of IFN-γ at days 5 and 10 post-*P. yoelii* infection. Administration of IFN-γ-neutralizing antibody significantly reduced the amount of serum IFN-γ compared to mice receiving control antibodies (Fig. 6a). Despite this reduction, malaria parasitemia was only slightly enhanced in this group (Fig. 6b) and fatalities did not occur.

### 4. Discussion

Previously, we reported that BALB/c mice concurrently infected with the intestinal trematode *E. caproni* and the normally non-lethal rodent malaria *P. yoelii* were susceptible to enhanced malaria parasitemia and death during co-infection (Noland et al., 2005). IFN-γ plays a central role in controlling blood stage malaria infection (Li et al., 2001), and mice unable to produce this cytokine are likewise susceptible to hyper-parasitemia and death (Su and Ste-
The goal of the present studies was to determine the effect of intestinal trematode and nematode infections on IFN-γ and other key cytokines during malaria co-infection in the \textit{P. yoelii–E. caproni} and \textit{P. yoelii–H. polygyrus} model systems. Previous studies have shown that a variety of inbred mouse strains mount a strong IFN-γ response to \textit{P. chabaudi} malaria infection, with peak activity occurring around day 5 post infection (Meding et al., 1990; Stevenson et al., 1990)—a result confirmed in this study with \textit{P. yoelii}. We therefore focused our attention on modulation of cytokine response during early malaria infection.

The present study demonstrated that chronic infection with either of two intestinal parasites, \textit{E. caproni} or \textit{H. polygyrus}, impaired peak IFN-γ production from polyclonally stimulated spleen cells of BALB/c mice during concurrent \textit{P. yoelii} infection. Helminth co-infected mice also displayed increased malaria parasitemia at day 10 post-malaria infection. Although this result was not statistically significant due to small samples sizes in the present study, previous results in the \textit{E. caproni–P. yoelii} model indicate that significant differences in malaria parasitemia were not observed until after day 14 of co-infection (Noland et al., 2005). A similar deficit in malaria-specific IFN-γ has been observed in \textit{P. chabaudi–H. polygyrus} co-infected C57BL/6 mice, which were also susceptible to hyper-parasitemia and death (Su et al., 2005). Taken together, these studies indicate that intestinal helminth infections in mice adversely affect host immune response to concurrent malaria infection, and furthermore suggest that the exacerbation of late-stage disease is associated with impairment of IFN-γ response early in infection.

In contrast to the findings of Su et al. (2005), we found no effect of concurrent helminth infection on humoral IFN-γ response (data not shown), suggesting that control of malaria may depend more critically on cellular INF-γ. In line with this hypothesis, we observed minimal exacerbation of disease when serum IFN-γ was neutralized through injection of antibodies throughout \textit{P. yoelii} infection. Such results are consistent with similar experiments in the \textit{P. chabaudi} model (Meding et al., 1990; Stevenson et al., 1990). Helminth species differ in their effect on host response to concurrent malaria infection. \textit{S. mansoni}-infected C57BL/6 mice, which...
were susceptible to enhanced *P. chabaudi* malaria, displayed only sporadic reduction in IFN-γ (Helmy et al., 1998), whereas levels of TNF-α, another Th1-associated cytokine implicated in control of rodent malaria infection (Jacobs et al., 1996), were significantly reduced. Other investigators have found that mice infected with either *S. mansoni* or the filarial nematode *Lumatodes signodontis* produced increased levels of IFN-γ during *P. chabaudi* infection (Graham et al., 2005; Yoshida et al., 2000). Such results may be explained by the fact that schistosomes and filarial worms exhibit both Th1 and Th2 properties (Maizels and Yazdanbakhsh, 2003), and effects of these tissue-borne parasites may be more heterogeneous than intestinal parasite infection.

In contrast to the Th1-associated protective immune response to malaria, helminth infection generally results in strong Th2 polarization, characterized by the production of IL-4, IL-5, IL-9, and IL-13, IgE antibodies, as well as subsequent activation of mast cells, eosinophils, and basophils (MacDonald et al., 2002). Indeed, *H. polygyrus* elicited high levels of IL-4 in these experiments, consistent with previous studies (Urban et al., 1991). *E. caproni* infection, however, resulted in relatively minor IL-4 production. This result is in agreement with a previous report of moderate IL-4 response to *E. caproni* infection in C57BL/6 mice (Brunet et al., 2000). The low level IL-4 response during *E. caproni* infection is remarkable as nearly all other helminths, including gastrointestinal nematodes (Finkelman et al., 1997), filarial nematodes (Lawrence et al., 1994), and *S. mansoni* (Pearce et al., 2004), induce a robust Th2 response during some or all stages of infection. It is likely that the exclusively enteric and non-invasive nature of the *E. caproni* life cycle in the mammalian host (Fried and Huffman, 1996) plays a major role in this finding, though a definitive mechanism has yet to be established. The *E. caproni* model thus offers a unique system in which the confounding effects of systemic tissue phase disease and gut invasion are eliminated from characterization of host immune response to intestinal parasites, and continued examination of this aspect should prove valuable for the immunology of echinostomes and helminths in general.

In this study, we observed a strong correlation between the relative production of IL-4 by helminth-infected mice and the magnitude of IFN-γ suppression during co-infection with *P. yoelii*. IL-4 directly suppresses Th1 activity and IFN-γ production (Seder et al., 1992; Skapenko et al., 2004), and previous work has also shown that this occurs in a dose dependent manner (Peleman et al., 1989). Th2 cells produce IL-4 under the control of the transcription factor GATA-3 (Murphy and Reiner, 2002). GATA-3 has already been implicated in Th1 down modulation in human helmint infections (Babu et al., 2006), and it would be interesting to evaluate its expression during malaria co-infection in the *E. caproni* and *H. polygyrus* models.

IL-10 also exerts potent anti-inflammatory properties (Groux et al., 1996) and plays a central role in regulating immune pathology to both malaria (Li et al., 1999) and helminth infections (Hoffmann et al., 2000). Indeed, in these studies, the down-regulation of IFN-γ in all malaria-infected groups by day 10 post-malaria infection was preceded by a vigorous IL-10 response in these groups at day 5. *H. polygyrus* infection alone also induced a significant IL-10 response, in agreement to previous findings (Su et al., 2005). However, as spleen cells from mice infected with *E. caproni* failed to produce levels of IL-10 greater than naïve uninfected mice, IL-10 production by spleen cells did not appear to be a causative factor in helminth-associated IFN-γ suppression. This does not exclude the possibilities, however, that IL-10 from other cellular sources such as dendritic cells or macrophages, or other regulatory factors play a role in these interactions. Indeed, recent studies have shown that *H. polygyrus* induces CD4+<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Finney et al., 2007) and CD8<sup>+</sup> regulatory T cells (Setiawan et al., 2007) that modify responses to non-parasite antigens and immune pathology at sites distal to worm infection. Exploration of the interaction of these cells with *Plasmodium* infection should prove fruitful.

Here, we were also able to examine the reciprocal effects of malaria infection on cytokine response to existing helminth infection. We found that the vigorous Th1 anti-malaria response down-regulated the established IL-4 response to both *E. caproni* and *H. polygyrus* infection. This also corroborates similar observations in the *H. polygyrus–P. chabaudi* system (Su et al., 2005). However, such modulation does not seem to alter viability or fecundity of *E. caproni*, as malaria had no effect on *E. caproni* fecal egg production during co-infection (Noland et al., 2005), and initiation of *E. caproni* infection simultaneous to, or during early malaria infection had no effect on worm establishment (data not shown). In contrast, concurrent *P. yoelii* infection has previously been found to enhance establishment of *S. mansoni* infection (Christensen et al., 1989).

In conclusion, these studies demonstrate that concurrent intestinal helminth and malaria infections can reciprocally regulate host immune response to either pathogen. Furthermore, findings presented here support the hypotheses that helminth-induced exacerbation of malaria is associated with a deficit in IFN-γ production, and that suppression of IFN-γ is related to IL-4 production during chronic helminth infection. Results from the co-infection models employed here provide insight into the complex interactions that have emerged from human co-infection studies, and continue to offer convenient systems to explore the mechanistic determinants.

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