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Impairment of Protective Immunity to Blood-Stage Malaria by Concurrent Nematode Infection

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Helminthiases, which are highly prevalent in areas where malaria is endemic, have been shown to modulate or suppress the immune response to unrelated antigens or pathogens. In this study, we established a murine model of coinfection with a gastrointestinal nematode parasite, *Heligmosomoides polygyrus*, and the blood-stage malaria parasite *Plasmodium chabaudi* AS in order to investigate the modulation of antimalarial immunity by concurrent nematode infection. Chronic infection with the nematode for 2, 3, or 5 weeks before *P. chabaudi* AS infection severely impaired the ability of C57BL/6 mice to control malaria, as demonstrated by severe mortality and significantly increased malaria peak parasitemia levels. Coinfected mice produced significantly lower levels of gamma interferon (IFN-γ) during *P. chabaudi* AS infection than mice infected with malaria alone. Concurrent nematode infection also suppressed production of type 1-associated, malaria-specific immunoglobulin G2a. Mice either infected with the nematode alone or coinfected with the nematode and malaria had high transforming growth factor β1 (TGF-β1) levels, and concurrent nematode and malaria infections resulted in high levels of interleukin-10 in vivo. Splenic $CD11c^+$ dendritic cells (DC) from mice infected with malaria alone and coinfected mice showed similarly increased expression of CD40, CD80, and CD86, but DC from coinfected mice were unable to induce $CD4^+$ T-cell proliferation and optimal IFN-γ production in response to the malaria antigen in vitro. Importantly, treatment of nematode-infected mice with an anthelmintic drug prior to malaria infection fully restored protective antimalarial immunity and reduced TGF-β1 levels. These results demonstrate that concurrent nematode infection strongly modulates multiple aspects of immunity to blood-stage malaria and consequently impairs the development of protective antimalarial immunity.

A number of factors have been proposed to be responsible for the failure to develop long-lasting immunity to natural malaria infection in areas of endemicity and for the difficulty of inducing strong protective immunity against malaria in vaccine field trials. These include the complex life cycle, antigenic diversity, and variation of malaria parasites, as well as genetic polymorphism and malnutrition of the human host and an immature immune system in children (16, 41). In addition, concurrent infection with helminth parasites, which are highly prevalent in many areas where malaria is endemic, has recently been recognized as a possible confounding factor modulating immune responses to other pathogens, including malaria parasites (31).

Malaria is highly endemic in sub-Saharan Africa, Southeast Asia, and South America, where there is also a high prevalence of helminth parasite infections. For example, infections with the major human gastrointestinal nematodes, including *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworm species *Ancylostoma duodenale* and *Necator americanus*, are widespread in most of these areas. The combined prevalence of infections with these gastrointestinal nematodes can be as high as 90% in some parts of the African continent (7, 8, 12). The situation of helminth infection in humans appears even more severe if other helminthiases, such as lymphatic filariasis and schistosomiasis, are included.

Modulation of immune responses to viral, bacterial, and protozoan pathogens by concurrent helminth infection has been observed in many human epidemiological studies and in laboratory animal models. Patients with helminth infections have been observed to have higher loads of human immunodeficiency virus in plasma and reduced delayed-type hypersensitivity responses to *Mycobacterium tuberculosis* purified protein derivative or house dust mite antigen (Ag) (4, 50). Human subjects infected with the filarial parasite *Onchocerca volvulus* have been observed to produce significantly lower levels of antitetanus antibody (Ab) following tetanus vaccination (9). In laboratory animal studies, mice coinfected with *Schistosoma mansoni* and *Leishmania major* show impaired ability to resolve *L. major* infection (23). Similar impairment of protective immunity by concurrent helminth infection has also been observed in other coinfection models, such as the nematode *Nippostrongylus brasiliensis* and the bacterium *Chlamydophila abortus* (5), the cestode *Taenia crassiceps* and the protozoan *Trypanosoma cruzi* (39), and *S. mansoni* and recombinant vaccinia virus (1). For malaria, it has been reported that mixed *Plasmodium falciparum* and *Plasmodium vivax* infections are more frequent in *A. lumbricoides*-infected patients in Thailand (32). Epidemiological studies also showed that worm infection in humans alters the development of cerebral malaria (33).

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Mice coinfected with *S. mansoni* and *Plasmodium chabaudi* develop increased malaria parasitemia (17).

To understand the impact of concurrent helminth parasite infection on the development of protective immunity to malaria, we established a mouse model of coinfection with the rodent blood-stage malaria parasite *P. chabaudi* AS and a murine nematode, *Heligmosomoides polygyrus*. *H. polygyrus* is a murine nematode parasite that dwells in the small intestine of the host. In most inbred strains of mice, *H. polygyrus* establishes a chronic primary infection, which lasts for several months (3, 30). Infection with this nematode parasite induces a strong Th2 immune response characterized by production of the cytokines interleukin-4 (IL-4), IL-5, and IL-13, immunoglobulin E (IgE) and IgG1 antibodies, eosinophilia, and mastocytosis in intestinal mucosal tissue (14, 15, 20, 22). In the present study, we observed that concurrent *H. polygyrus* infection rendered otherwise resistant C57BL/6 (B6) mice highly susceptible to blood-stage *P. chabaudi* AS infection. Furthermore, coinfection of malaria-infected mice with a nematode severely impaired the development of protective immunity against malaria by altering a number of key immune responses.

MATERIALS AND METHODS

Mice, parasites, and experimental infection. Age- and sex-matched mice, 6 to 8 weeks old, were used in all experiments. B6 and BALB/c mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the animal facility of the Montreal General Hospital Research Institute (Montreal, Quebec, Canada) under specific-pathogen-free conditions. Blood-stage *P. chabaudi* AS malaria parasites were maintained in A/J mice by weekly passage as described elsewhere (37). Infections were initiated by intraperitoneal (i.p.) injection of 10^6 *P. chabaudi* AS-parasitized red blood cells (pRBC). Parasitemia of individual mice was monitored on blood smears stained with Diff-Quik (American Scientific Products, McGraw Park, IL). *H. polygyrus* was kindly provided by M. E. Scott (McGill University, Montreal, Quebec, Canada). To maintain and propagate *H. polygyrus*, BALB/c mice were inoculated with 400 third-stage larvae (L3) by oral gavage with a blunt needle. Feces containing *H. polygyrus* eggs were collected from the mice 20 to 25 days after infection and cultured on moist filter paper in petri dishes for 7 days at room temperature. The L3 hatched and released from the eggs were harvested, washed, and stored in water at 4°C. For experimental infection, mice were infected with 200 L3 by oral inoculation.

Spleen cell cultures. Spleens from normal and infected mice were removed aseptically. Single-cell suspensions were prepared in RPMI 1640 medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT), 25 mM HEPES (Life Technologies), 0.12% gentamicin (Schering, Montreal, Quebec, Canada), and 2 mM glutamine (Life Technologies) (complete medium). Red blood cells were lysed with 0.175 M NH_4Cl. Cell suspensions were filtered through a cell strainer (Fisher Scientific, Nepean, Ontario, Canada). The viability of the cells was determined by trypan blue exclusion and was always >95%. Aliquots of 1-ml cell suspensions at a concentration of 5×10^6 cells/ml were cultured in triplicate in 48-well tissue culture plates in complete medium as a control or in medium containing pRBC (10^6/ml) as a source of malaria antigen or *H. polygyrus* adult worm Ag (Hp-Ag) (20 μg/ml). The cultures were incubated for 48 h at 37°C in a humidified CO_2 incubator. Supernatants were collected and stored at −20°C until assayed for cytokine levels. To prepare Hp-Ag, BALB/c mice were infected with 400 to 500 *H. polygyrus* L3 and sacrificed 21 days postinfection. *H. polygyrus* adult worms were collected from the small intestine, washed extensively with sterile phosphate-buffered saline, and cultured overnight in RPMI 1640 medium containing antibiotics. The adult worms were homogenized with a glass tissue grinder, and the homogenate was centrifuged ($3{,}000 \times g$, 10 min). The soluble fraction of the homogenate was collected, the protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA), and the material was stored at −20°C.

Cytokine ELISAs. Levels of gamma interferon (IFN-γ), interleukin-4 (IL-4), and IL-10 in cell culture supernatants and serum samples were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using paired capture and detection Abs as previously described (46, 47). The concentrations of cytokines in the samples were calculated against a standard curve generated with recombinant cytokines (BD PharMingen, San Diego, CA). For detecting transforming growth factor β1 (TGF-β1) in plasma, normal and infected mice were bled by cardiac puncture and blood was collected into EDTA-coated tubes (Becton Dickinson, Franklin Lakes, NJ) bathed in ice. The tubes were immediately centrifuged at $250 \times g$ for 10 min, and the cell-free fraction was collected and further centrifuged at $750 \times g$ for 15 min to obtain platelet-depleted plasma (36). The plasma samples were collected and stored at −70°C. The levels of TGF-β1 in plasma were measured by an ELISA using paired capture and detection rat anti-TGF-β1 monoclonal Abs (MAbs) (BD PharMingen). Briefly, Immulon II plates (Dynatech, Chantilly, VA) were coated with an anti-TGF-β1 capture MAb (clone A75-2.1) overnight at 4°C and subsequently blocked with 1% bovine serum albumin in phosphate-buffered saline for 3 h. To detect total (latent plus bioactive) TGF-β1, the plasma samples were acidified with 1 N HCl at room temperature for 20 min, followed by neutralization with 1 N NaOH, before they were assayed by ELISA. To measure bioactive TGF-β1, plasma samples were directly added to the ELISA plate. Plates were incubated overnight at 4°C. After extensive washing, a biotin-conjugated anti-TGF-β1 MAb (clone A75-3.1) and a streptavidin-horseradish peroxidase (HRP) conjugate were added sequentially, followed by incubation at room temperature for 3 h. Reactivity was visualized using ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] as the substrate, and optical density values were read at 405 nm with a reference wavelength of 492 nm. Human recombinant TGF-β1 (eBioscience, San Diego, CA) was used for generation of a standard curve.

Isolation of splenic DC and $CD4^+$ T cells and coculture. Spleens from normal and infected mice were removed aseptically. Single-cell suspensions were prepared by collagenase digestion as described by others (19). Low-density cells were separated by density gradient centrifugation using NycoPrep (Axis-Shield, Oslo, Norway), and $CD11c^+$ dendritic cells (DC) were isolated from the low-density cells by positive selection with anti-CD11c MAb-conjugated magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The enriched DC were >90% positive for CD11c as determined by flow cytometry. Splenic DC were doubly stained with a fluorescein isothiocyanate-conjugated anti-CD11c MAb and a phycoerythrin-conjugated MAb against CD40, CD80, or CD86 (BD PharMingen), and the expression of costimulatory molecules was analyzed by flow cytometry. To isolate $CD4^+$ T cells, spleens were collected from normal mice and single-cell suspensions were prepared as described above. $CD4^+$ T cells were isolated with anti-CD4 MAb-conjugated magnetic beads (Miltenyi Biotec). The enriched $CD4^+$ T cells had >92% purity. For stimulation of $CD4^+$ T cells by splenic DC, 1×10^5 purified $CD4^+$ T cells were cocultured with various numbers of purified DC (DC:T-cell ratio, 1:4 to 4:4) in the presence of pRBC (1×10^6/ml) in triplicate in a U-bottom 96-well cell culture plate for 48 h. During the last 6 h of culture, 1 μCi/well of [^{3}H]thymidine was added. Cells were harvested, and incorporation of [^{3}H]thymidine by proliferating cells was determined by scintillation counting. To determine the ability of DC to induce cytokine production by $CD4^+$ T cells, 4×10^5 enriched $CD4^+$ T cells were cultured with 2×10^5 splenic DC in a 96-well cell culture plate for 48 h in the presence of either 1×10^6 pRBC/ml or 20 μg/ml Hp-Ag. Cell culture supernatants were harvested, and cytokine levels were determined by ELISA as described above.

***P. chabaudi* AS-specific antibody levels.** Serum levels of *P. chabaudi* AS-specific antibody isotypes were determined by ELISA as described elsewhere (46). For detecting total Ig and IgG1, HRP-conjugated goat anti-mouse Ig and IgG1 Abs (Southern Biotechnology Associates, Inc., Birmingham, AL) were used, respectively. To measure the IgG2a subclass in B6 mice, an HRP-conjugated goat polyclonal Ab specific to the $IgG2a^b$ allotype was used as the detecting Ab (Southern Biotechnology Associates, Inc.) (29). Antibody levels in serum are expressed as end point titers, the reciprocal of the lowest dilution that yields the background optical density.

Anthelmintic drug treatment. To terminate *H. polygyrus* infection, mice were treated orally with pyrantel pamoate (100 mg/kg body weight) (kindly provided by M. Gottschalk, Université de Montréal, St-Hyacinthe, Quebec, Canada) to remove adult *H. polygyrus* parasites (22).

Statistical analysis. For analyzing parasitemia data, a repeated-measures analysis of variance was performed followed by Dunnett's multiple comparison test to determine the significance of the effect of nematode coinfection on peak parasitemia levels. Cytokine and antibody data are presented as the median and individual data or the median with minimum and maximum values for each group. A nonparametric Mann-Whitney U test was used to test the significance of differences between experimental groups. A P value less than 0.05 was considered significant.

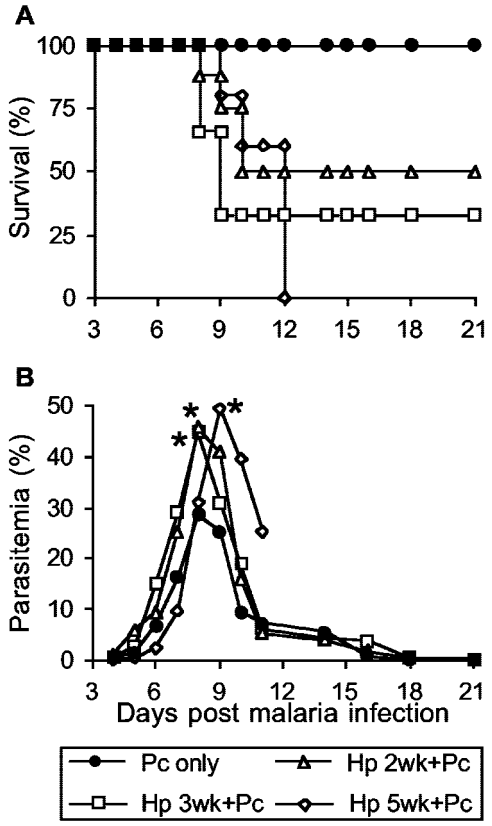

FIG. 1. Mortality and levels of parasitemia in B6 mice infected with blood-stage *P. chabaudi* AS (Pc) or coinfected with the nematode *H. polygyrus* (Hp) and malaria. Groups of B6 mice ($n = 5$) were infected by oral inoculation with 200 *H. polygyrus* L3 for 2, 3, or 5 weeks. Nematode-infected mice and a group of normal mice were infected i.p. with 10^6 *P. chabaudi* AS-parasitized RBC. Mortality (A) and malaria parasitemia (B) were monitored for 3 weeks after malaria infection. Mortality is calculated from pooled data ($n = 13$ to 15) from three experiments. Parasitemia results shown are from one of three experiments. Asterisk indicates significant difference ($P < 0.05$) in peak parasitemia between coinfected and singly malaria infected groups.

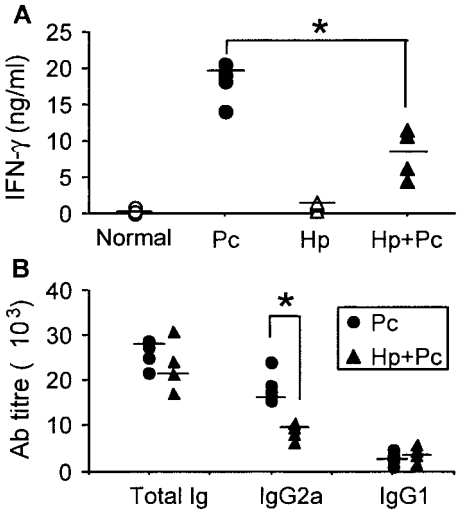

FIG. 2. Levels of IFN-γ and *P. chabaudi* AS-specific antibody in sera. (A) Sera were collected from normal uninfected B6 mice, from mice singly infected with either 200 *H. polygyrus* (Hp) L3 (20 days postinfection) or 10^6 *P. chabaudi* AS (Pc)-parasitized RBC (6 days postinfection), and from mice coinfected with *H. polygyrus* (20 days) and *P. chabaudi* AS (6 days). IFN-γ levels were determined by ELISA. (B) B6 mice were either singly infected with *P. chabaudi* AS or coinfected with *H. polygyrus* and *P. chabaudi* AS, and serum samples were collected 5 weeks post-*P. chabaudi* AS infection. Levels of *P. chabaudi* AS-specific total Ig, IgG1, and IgG2a were measured by ELISA. Data from individual mice and the median for each group ($n = 4$) are presented. Results shown are representative of four (A) and two (B) independent experiments. Asterisk indicates a significant difference between groups ($P < 0.05$).

RESULTS

Impairment of antimalarial immunity by concurrent nematode infection. We performed a series of *H. polygyrus* and *P. chabaudi* AS coinfection experiments to determine whether concurrent nematode infection impairs the development of protective immunity to a primary malaria infection. Female B6 mice were infected orally with 200 *H. polygyrus* L3 for either 2, 3, or 5 weeks. The *H. polygyrus*-infected mice and a group of uninfected normal mice were infected i.p. with 1×10^6 pRBC to initiate blood-stage malaria infection. Mortality and malaria parasitemia were monitored for 3 weeks. Single infection with *H. polygyrus* (data not shown) or blood-stage *P. chabaudi* AS did not result in any mortality in B6 mice (Fig. 1A). However, B6 mice infected with *H. polygyrus* for 2, 3, or 5 weeks had 50%, 70%, or 100% mortality, respectively, following *P. chabaudi* AS infection (Fig. 1A). In addition, the nematode- and malaria-coinfected mice developed increased levels of malaria parasitemia (Fig. 1B). The peak parasitemia levels reached in nematode- and malaria-coinfected mice at days 8 to 9 after *P. chabaudi* AS infection were significantly higher than those of control mice (all $P < 0.05$). B6 mice infected with *H. polygyrus* 1 week prior to malaria infection also had significantly higher levels of parasitemia than control mice, but no mortality occurred (data not shown). Interestingly, mice in which coinfection was initiated on the same day had similar levels of parasitemia as the malaria control group (data not shown), suggesting that establishment of a chronic nematode infection of at least 1 week's duration is necessary for suppression of antimalarial immunity. These results demonstrate that concurrent nematode infection impaired the development of protective immunity to primary blood-stage malaria infection. For all subsequent experiments, infection with *H. polygyrus* for 2 weeks prior to *P. chabaudi* AS infection was chosen as the coinfection protocol, except where otherwise indicated.

Cytokine and malaria-specific antibody responses in coinfected mice. It has been shown previously that IFN-γ is required for control of acute blood-stage *P. chabaudi* AS infection (43, 44, 46). To determine whether production of this protective cytokine is modulated by concurrent nematode infection, we analyzed IFN-γ responses in vivo in nematode or malaria singly infected and coinfected mice. As observed in our previous studies (44, 47), B6 mice produced a high level of IFN-γ in vivo in response to blood-stage *P. chabaudi* AS infection, while *H. polygyrus* infection did not induce significant IFN-γ production (Fig. 2A). Importantly, IFN-γ levels in the sera of nematode- and malaria-coinfected mice were signifi-

cantly reduced from those in mice infected with *P. chabaudi* AS alone.

We also examined the effect of concurrent nematode infection on the development of malaria-specific antibody responses. Sera were collected from singly malaria infected and nematode- and malaria-coinfected mice 5 weeks after malaria infection, and *P. chabaudi* AS-specific total Ig, IgG2a, and IgG1 antibody levels were determined. As shown in Fig. 2B, the two groups of mice produced similar levels of total Ig while low levels of IgG1 were detected in both groups. Consistent with the significantly lower serum levels of the type-1 cytokine IFN-γ, coinfected mice produced significantly lower levels of malaria-specific IgG2a antibody than mice infected with malaria alone.

To determine pathogen-specific type-1 and type-2 cytokine production, spleen cells from normal mice, mice singly infected with *H. polygyrus* or *P. chabaudi* AS, and coinfected mice were cultured in vitro and stimulated with pRBC or Hp-Ag. Spleen cells from normal control mice and from mice infected with *H. polygyrus* produced low levels of IFN-γ in response to stimulation with either antigen (Fig. 3A). However, spleen cells from *P. chabaudi* AS-infected mice produced high levels of IFN-γ when stimulated with pRBC. Cells from coinfected mice produced significantly lower levels of IFN-γ in response to stimulation with pRBC than cells from mice infected with *P. chabaudi* AS alone (Fig. 3A).

Consistent with the results of previous studies (14, 22), spleen cells from mice infected with *H. polygyrus* that were stimulated with Hp-Ag produced high levels of IL-4, which peaked 2 weeks after infection (Fig. 3B). Infection with blood-stage *P. chabaudi* AS does not induce increased production of IL-4 in B6 mice during the first 3 weeks of infection (44, 47). Spleen cells from mice infected 6 days previously with *P. chabaudi* AS or from coinfected mice produced only basal or low levels of IL-4 in response to stimulation with pRBC (Fig. 3C). However, stimulation with Hp-Ag induced high levels of IL-4 production by spleen cells from singly nematode infected mice as well as from nematode- and malaria-coinfected mice (Fig. 3C).

The immunoregulatory cytokines TGF-β and IL-10 have been shown to suppress protective immunity to a number of infections, including malaria (35, 38, 48). Thus, we measured the in vivo levels of TGF-β1 and IL-10 during *H. polygyrus* and *P. chabaudi* AS single infections. Since TGF-β is known to occur in latent and bioactive forms (24, 35), TGF-β1 levels were quantified with (latent plus bioactive) and without (bioactive) acidification of plasma samples prior to ELISA. It was observed that all samples had high levels of total TGF-β1 (1.8 to 2.3 ng/ml). No significant differences in total TGF-β1 levels were detected between infection groups or between different time points of infection (data not shown). The level of bioactive TGF-β1 was not changed during the first week of blood-stage malaria infection but was increased significantly from day 9 to 14 after *P. chabaudi* AS infection and declined to the basal level with the resolution of malaria 4 weeks after infection (Fig. 4A). In contrast, infection with *H. polygyrus* induced remarkably high levels of bioactive TGF-β1. The level was increased significantly from day 9 after nematode infection and continued to increase with the progression of infection, reaching a peak of approximately 600 pg/ml by 4 weeks after infection

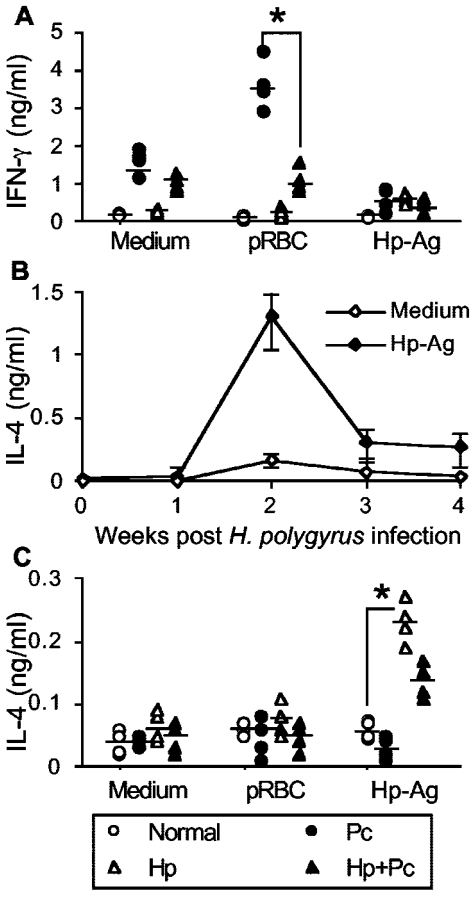

FIG. 3. IFN-γ and IL-4 production by spleen cells in vitro. (A) Spleens were collected from normal uninfected B6 mice, from mice singly infected with either 200 *H. polygyrus* (Hp) L3 (20 days postinfection) or 10^6 *P. chabaudi* AS (Pc)-parasitized RBC (6 days postinfection), and from mice coinfected with *H. polygyrus* (20 days) and *P. chabaudi* AS (6 days). Spleen cells were cultured as described in Materials and Methods. IFN-γ levels in supernatants were determined by ELISA. Data from individual mice and the median for each group ($n = 4$) are presented. (B) Groups of B6 mice were infected with 200 *H. polygyrus* L3 for the indicated times. Spleen cells were cultured in medium or in the presence of Hp-Ag, and IL-4 levels in supernatants were measured by ELISA. Data are presented as medians, with minimum and maximum values ($n = 4$) indicated by error bars for each group. (C) Spleens were collected from the four groups of mice, and cells were cultured as described for panel A. IL-4 levels in supernatants were measured by ELISA. Data from individual mice and the median for each group ($n = 4$) are presented. Results shown are representative of two experiments. Asterisk indicates a significant difference ($P < 0.05$) between groups.

(Fig. 4B). Mice coinfected with the nematode and malaria had significantly higher levels of bioactive TGF-β1 than mice infected with malaria alone (Fig. 4C). In addition, B6 mice had increased levels of serum IL-10 from day 6 to 14 following blood-stage *P. chabaudi* AS infection (Fig. 4D and data not shown). In B6 mice infected with *H. polygyrus*, a small increase in IL-10 levels was detected during the first 4 weeks of infection (Fig. 4D and data not shown). Importantly, mice coinfected with *H. polygyrus* and *P. chabaudi* AS produced significantly higher levels of IL-10 than mice singly infected with either pathogen (Fig. 4D).

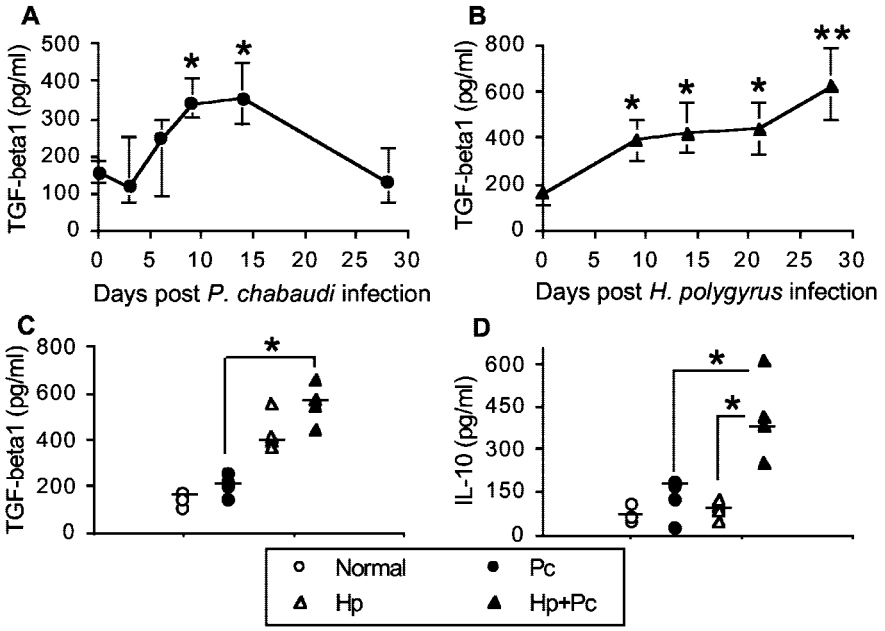

FIG. 4. Levels of bioactive TGF-β1 in plasma and of IL-10 in serum. (A and B) Plasma samples were collected from B6 mice at the indicated times after blood-stage *P. chabaudi* AS (A) or *H. polygyrus* (B) single infection, and levels of bioactive TGF-β1 were determined by ELISA. Data are presented as medians, with minimum and maximum values ($n = 4$) indicated by error bars for each group. Single ($P < 0.05$) and double ($P < 0.01$) asterisks indicate significant differences from values for normal uninfected mice. (C and D) Plasma or serum samples were collected from normal uninfected B6 mice, from mice singly infected with either *H. polygyrus* (Hp; 20 days postinfection) or *P. chabaudi* AS (Pc; 6 days postinfection), and from mice coinfected with *H. polygyrus* (20 days) and *P. chabaudi* AS (6 days), and levels of bioactive TGF-β1 in plasma (C) and IL-10 in serum (D) were determined by ELISA. Data from individual mice and the median for each group ($n = 4$) are presented. Asterisks ($P < 0.01$) indicate significant differences between groups. Results are representative of three (A and B) or two (C and D) experiments.

DC function in coinfected mice. To characterize DC maturation and function during malaria and nematode single infections and coinfection, we first analyzed the expression of costimulatory molecules by splenic $CD11c^+$ DC. DC from *H. polygyrus*-infected mice exhibited both percentages of $CD40^+$, $CD80^+$, and $CD86^+$ cells and levels of expression of these costimulatory molecules comparable to those of DC from uninfected control mice (Table 1). In contrast, DC from *P. chabaudi* AS-infected mice showed significantly elevated expression of these costimulatory molecules. The percentages of DC expressing CD80 and CD86, but not CD40, and the expression levels of all three costimulatory molecules were significantly increased in DC from malaria-infected mice compared with DC from normal mice. DC from the nematode- and malaria-coinfected mice also showed a pattern of significantly increased percentages and expression levels of CD40, CD80, and CD86 similar to that of DC from *P. chabaudi* AS singly infected mice (Table 1), suggesting that preinfection with the nematode parasite did not suppress the expression of costimulatory molecules by DC in response to malaria infection.

Coculture of enriched DC and $CD4^+$ T cells was performed to analyze the ability of DC from nematode- and malaria-infected mice to activate $CD4^+$ T cells. DC from normal uninfected control and *H. polygyrus* infected mice induced low levels of $CD4^+$ T-cell proliferation (Fig. 5). In contrast, DC from *P. chabaudi* AS-infected mice induced high levels of $CD4^+$ T-cell proliferation compared with DC from control or nematode-infected mice. In comparison with DC from singly malaria infected mice, DC from coinfected mice induced significantly lower levels of $CD4^+$ T-cell proliferation at the higher DC/$CD4^+$ T-cell ratios (Fig. 5).

Bone marrow-derived DC primed in vitro with helminth

TABLE 1. Expression of costimulatory molecules by splenic $CD11c^+$ DC[a]

Group	CD40		CD80		CD86	
	% $CD11c^+$ DC	MFI	% $CD11c^+$ DC	MFI	% $CD11c^+$ DC	MFI
Normal	75.5 (74.2–77.4)	124 (111–131)	76.1 (72.3–76.6)	184 (180–192)	56.2 (53.1–57.2)	156 (126–167)
Hp infected	78.7 (74.2–78.9)	161 (134–164)	77.2 (76.8–78.4)	213 (206–240)	52.6 (50.5–54.8)	144 (144–151)
Pc infected	68.2 (64.4–74.3)	791**[b] (770–808)	87.4* (84.9–89.1)	687** (626–691)	86.3* (80.5–87.1)	238* (229–264)
Hp + Pc	77.6 (75.9–79.2)	721* (715–733)	80.4* (79.4–84.1)	621** (584–655)	80.7* (79.3–83.9)	238* (212–241)

[a] $CD11c^+$ DC were isolated by magnetic sorting from spleens of normal, *P. chabaudi* AS (Pc)-infected (3 days), *H. polygyrus* (Hp)- infected (17 days), and Hp (17 days)- and Pc (3 days)-coinfected (Hp + Pc) mice. Percentages of DC expressing CD40, CD80, and CD86 and their expression levels (estimated by mean fluorescence intensity [MFI]) were determined by flow cytometry. Results are presented as medians with minimum and maximum values for each group ($n = 3$). Results are representative of two independent experiments.

[b] *, $P < 0.05$; **, $P < 0.01$. Significance levels refer to comparison with DC from normal mice.

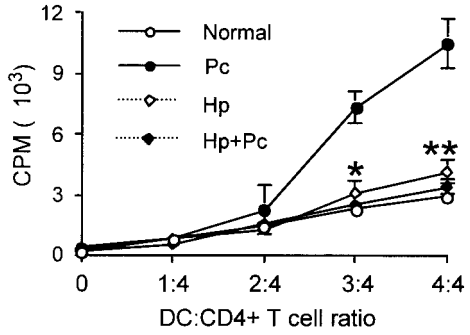

FIG. 5. Proliferation of enriched $CD4^+$ T cells induced by splenic $CD11c^+$ DC. $CD11c^+$ DC were isolated by magnetic sorting from spleens of normal, *P. chabaudi* AS (Pc)-infected (3 days), *H. polygyrus* (Hp)-infected (17 days), and coinfected B6 mice and were then cocultured with $CD4^+$ T cells isolated from spleens of normal mice in the presence of 10^6 pRBC/ml for 48 h. During the last 6 h of culture, 1 μCi/well of [^{3}H]thymidine was added, and incorporation of [^{3}H]thymidine was determined. Single ($P < 0.05$) and double ($P < 0.01$) asterisks indicate significant differences between DC from coinfected mice and singly *P. chabaudi* AS infected mice.

parasite Ag or bacterial Ag induce distinct patterns of cytokine production by $CD4^+$ T cells when transferred to naïve mice (26). To determine if the phenotype of DC was modulated differently during nematode versus malaria infection, splenic DC isolated from normal and infected mice were cultured alone or cocultured with $CD4^+$ T cells from normal mice, and production of IFN-γ and IL-4 was analyzed. When cultured alone, DC from normal, malaria or nematode singly infected, and coinfected mice produced low basal levels of IFN-γ and IL-4 (data not shown). In the absence of antigen stimulation, DC from normal and *H. polygyrus*-infected mice induced low levels of IFN-γ production by naïve $CD4^+$ T cells (Table 2). However, DC from *P. chabaudi* AS-infected mice induced a high level of IFN-γ production in the absence of Ag, and this IFN-γ response was enhanced when pRBC were added to the culture. In the presence of pRBC, DC from coinfected mice also induced IFN-γ production by $CD4^+$ T cells, but the level was significantly lower than that induced by DC from *P. chabaudi* AS singly infected mice. DC from *H. polygyrus*-infected mice and coinfected mice stimulated $CD4^+$ T cells to produce IL-4 in response to Hp-Ag stimulation.

Deworming restores antimalarial immunity. It was of interest to determine if deworming prior to malaria infection was effective in restoring antimalarial immunity. Two groups of B6 mice were infected with *H. polygyrus*, and 2 weeks later, one group was treated with the anthelmintic drug pyrantel pamoate to terminate nematode infection. In addition, a group of normal mice was treated with the drug to control for the effect of the anthelmintic drug on malaria infection. One week after drug treatment, these three groups of mice and a group of naïve mice were infected with blood-stage *P. chabaudi* AS, and malaria parasitemia and mortality were monitored. Normal mice, either untreated or treated with the anthelmintic drug 1 week before malaria infection, showed similar levels of parasitemia following *P. chabaudi* AS infection (Fig. 6A). Mice preinfected with *H. polygyrus* but not treated with the drug developed significantly higher levels of peak parasitemia, with 30% mortality following malaria infection, compared to the control group infected with *P. chabaudi* AS alone. Termination of nematode infection prior to malaria infection significantly reduced the levels of parasitemia in comparison with those for the *H. polygyrus*-infected mice that were not drug treated (Fig. 6A), and 100% of drug-treated mice survived malaria infection. As described above, *H. polygyrus*-infected mice produced high levels of bioactive TGF-β1 in vivo. Removal of the nematode by anthelmintic drug treatment significantly reduced the levels of this cytokine (Fig. 6B).

DISCUSSION

Despite the continuous effort to control and eradicate helminth parasites in human populations during the past century, infections with these parasites are still highly prevalent in many parts of the world, including areas where malaria is endemic. Helminth infections not only cause direct adverse effects on human health, but studies with both human and laboratory animals demonstrate that they also modulate, in most cases suppressing, the protective immune response to infections caused by other pathogens including viruses, bacteria, and protozoan parasites (4, 9, 39, 50). Epidemiological studies also suggest that concurrent gastrointestinal nematode infections increase the susceptibility of humans to malaria (31, 32). We established a murine model of nematode and blood-stage malaria coinfection and demonstrated here the pronounced and detrimental effects of concurrent nematode infection on the development of protective immunity against malaria. Resistant B6 mice infected with blood-stage *P. chabaudi* AS develop a moderate level of acute malaria parasitemia and control and resolve the infection within 4 to 5 weeks; 100% survive. However, infection of B6 mice with *H. polygyrus* 2 to 5 weeks prior

TABLE 2. Cytokine production in cocultures of DC and $CD4^+$ T cells[a]

Stimulus	Production (ng/ml) of the following cytokines by DC cells from the indicated group:							
	IFN-γ				IL-4			
	Normal	Pc infected	Hp infected	Hp + Pc	Normal	Pc infected	Hp infected	Hp + Pc
Medium	1.4 (0.9–1.5)	7.5 (6.4–8.2)	1.2 (0.8–1.5)	6.2 (5.8–7.5)	0.14 (0.08–0.14)	0.19 (0.10–0.31)	0.37 (0.29–0.41)	0.21 (0.15–0.29)
pRBC	1.6 (1.3–2.5)	16.3 (14.9–18.1)	1.4 (1.1–1.9)	13.9*[b] (12.6–14.2)	0.12 (0.03–0.16)	0.21 (0.14–0.26)	0.40 (0.32–0.46)	0.48 (0.33–0.56)
Hp-Ag	1.2 (0.8–1.5)	6.9 (5.8–7.8)	0.5 (0.3–0.9)	1.9 (3.0–1.6)	0.18 (0.11–0.24)	0.32 (0.22–0.41)	1.91 (1.44–2.31)	1.76 (1.33–2.12)

[a] $CD11c^+$ DC were isolated by magnetic sorting from spleens of normal, *P. chabaudi* AS (Pc)-infected (3 days), *H. polygyrus* (Hp)-infected (17 days), and coinfected (Hp + Pc) mice and were cocultured with $CD4^+$ T cells enriched from spleens of normal mice for 48 h in medium alone or in the presence of pRBC or Hp-Ag. Cytokine levels in supernatants were determined by ELISA. Data are presented as medians with minimum and maximum values for each group ($n = 3$). Results are representative of two experiments.

[b] *, $P < 0.05$ in comparison with DC from *P. chabaudi* AS singly infected mice.

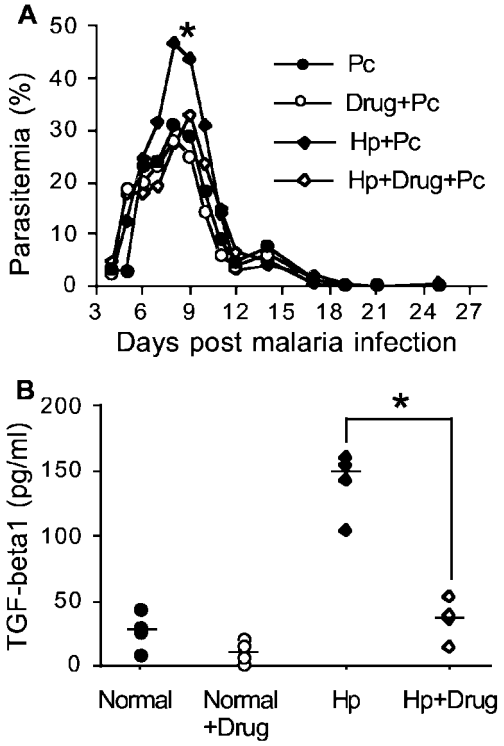

FIG. 6. Effects of deworming on antimalarial immunity and TGF-β1 levels in coinfected mice. (A) Two groups of B6 mice ($n = 5$) were infected with 200 *H. polygyrus* (Hp) L3. Two weeks after *H. polygyrus* infection, one group of *H. polygyrus*-infected mice and one group of normal mice were treated with pyrantel pamoate. One week after drug treatment, the three groups, as well as a group of normal mice, were infected with blood-stage *P. chabaudi* AS (Pc), and malaria parasitemia was determined. Asterisks ($P < 0.05$) indicate a significant difference in peak parasitemia from mice infected with *H. polygyrus* and treated with drug. (B) Four groups of B6 mice were infected with *H. polygyrus* and drug treated as described for panel A. All animals were sacrificed 1 week after drug treatment, and levels of bioactive TGF-β1 in plasma were determined. Data from individual mice and the median for each group ($n = 4$) are presented. Asterisks ($P < 0.05$) indicate significant differences between groups.

to *P. chabaudi* AS infection resulted in a more severe course of malaria. Nematode- and malaria-coinfected mice had high mortality following blood-stage *P. chabaudi* AS infection and developed significantly higher levels of peak malaria parasitemia than control mice singly infected with *P. chabaudi* AS. A similar impairment of protective immunity to blood-stage *P. chabaudi* AS was also observed in mice coinfected with *S. mansoni* (17). Together, these results indicate that concurrent helminth parasite infection severely impairs the ability of mice to control blood-stage malaria infection. The nematode-induced immunosuppression appears to be independent of the gender as well as the genetic background of the host: similar impairment of protective immunity to malaria by concurrent nematode infection was observed in female as well as male B6 mice and in female BALB/c mice (unpublished observations). Importantly, we also observed that treatment with an anthelmintic drug to remove nematode parasites before *P. chabaudi* AS infection restored immunity to malaria. This observation suggests that nematode-induced immunosuppression is not permanent and requires the presence of a chronic nematode infection.

The type-1-associated cytokines IL-12 and IFN-γ have been shown to play pivotal roles in control of primary blood-stage malaria in mice (44–47). Infection with blood-stage *P. chabaudi* AS in resistant B6 mice induces strong IL-12 and IFN-γ responses (44, 46). B6 mice lacking the IL-12 p40 or IFN-γ gene or treated with a neutralizing anti-IL-12 or anti-IFN-γ MAb develop more-severe courses of *P. chabaudi* AS infection (45–47). Consistent with these observations, we observed a strong in vivo IFN-γ response in B6 mice during the first week after *P. chabaudi* AS infection, and spleen cells from these mice produced high levels of IFN-γ in vitro in response to stimulation with pRBC. However, the in vivo and in vitro IFN-γ responses, essential for control and resolution of primary blood-stage *P. chabaudi* AS infection, were significantly reduced in mice coinfected with nematodes and malaria parasites. The reduced IFN-γ production in coinfected mice is likely to be the primary reason for their impaired ability to control *P. chabaudi* AS infection.

We have previously demonstrated that the type-1 cytokine-dependent antibody isotype IgG2a plays an important role in antibody-mediated adaptive immunity against blood-stage *P. chabaudi* AS infection (47). In the present study, we observed that nematode- and malaria-coinfected mice produced significantly lower levels of malaria-specific IgG2a antibody but that the malaria-specific total Ig and type-2-associated IgG1 antibody levels were similar to the levels observed in mice singly infected with *P. chabaudi* AS. Despite reduced IgG2a production, coinfected mice that survived the acute phase of *P. chabaudi* AS infection were able to resolve the primary malaria infection as efficiently as mice singly infected with *P. chabaudi* AS. These results suggest that other immune effector mechanisms may also contribute to the resolution of primary malaria infection.

As one of the most potent antigen-presenting cell types, DC are capable of activating naïve $CD4^+$ T cells to initiate adaptive immunity. An important feature of DC is their plasticity (18). Upon exposure to different pathogens or Ags, DC undergo maturation to acquire distinct phenotypes and induce either type-1 or type-2 polarization of immune responses (6, 26, 42). For example, bone marrow-derived DC primed with *S. mansoni* egg antigen exhibit a phenotype that is similar to that of immature DC and stimulate $CD4^+$ T cells to produce high levels of type-2 cytokines. In contrast, DC pulsed with the intracellular bacterium *Propionibacterium acnes* induce a type-1 cytokine response (6, 26). Here, we observed that blood-stage malaria infection induced increased expression of the costimulatory molecules CD40, CD80, and CD86 by splenic DC. In contrast, DC from the spleens of mice infected with *H. polygyrus* did not show enhanced expression of these costimulatory molecules. Interestingly, DC from nematode- and malaria-coinfected mice exhibited a pattern of increased expression of costimulatory molecules comparable to that observed in DC from singly malaria infected mice. These results suggest that a chronic infection with nematodes did not suppress the maturation of DC in response to malaria infection. However, the ability of splenic DC from coinfected mice to activate $CD4^+$ T cells was severely impaired in comparison with that of DC from mice infected with *P. chabaudi* AS alone. Despite low

levels of costimulatory molecule expression and an impaired ability to activate malaria-specific $CD4^+$ T cells, DC from coinfected mice as well as from *H. polygyrus* singly infected mice were able to induce production of the type-2 cytokine IL-4 by $CD4^+$ T cells in response to nematode antigen.

Infection with parasitic helminths in both human and laboratory animals generally induces a type-2 immune response characterized by production of IL-4, IL-5, and IL-13, IgE and IgG1 antibody production, and eosinophilia (10, 14, 27, 28). Consistent with these observations, spleen cells from mice infected with *H. polygyrus* produced high levels of IL-4, which contribute to protection against this parasite (49). On the other hand, protection against intracellular pathogens requires cell-mediated immunity promoted by a type-1 cytokine response. Because of the mutually antagonistic effects between type-1 and type-2 cytokines, it is generally believed that the type-2 cytokines produced during helminth infection may inhibit a type-1 immune response and, consequently, impair immune protection against intracellular pathogens (11). However, we have observed that *H. polygyrus*- and *P. chabaudi* AS-coinfected STAT-6 gene knockout mice, which are able to produce IL-4 and IL-13 in response to *H. polygyrus* infection (15) but have disrupted signaling pathways for both cytokines (21), developed a more severe course of malaria with high mortality, similar to that of coinfected wild-type B6 mice (unpublished observations). This result suggests that type-2 cytokines induced by the nematode *H. polygyrus* may not be the principal factor responsible for the impaired protective immunity to malaria in coinfected mice.

Most helminth parasites are long-lived and, in general, cause chronic infections (3, 30). However, the immune mechanism(s) underlying the chronicity of helminth infection is not fully understood. IL-10 and TGF-β1 are two important immunoregulatory cytokines. TGF-β1 has been shown to inhibit protective immune responses to infections with a number of intracellular pathogens, including *Plasmodium* (35, 36, 38, 48). Chronic filarial infections in humans and laboratory animals have been shown to induce increased production of IL-10 and TGF-β by regulatory T cells, which is thought to facilitate the chronic course of filarial infection (2, 13, 34, 40). Taken together, these results suggest that immunosuppressive cytokines induced during helminth infection mediate helminth-associated immunosuppression. In the present study, we observed that B6 mice infected with the gastrointestinal nematode *H. polygyrus* produced high levels of bioactive TGF-β1 in vivo 2 to 3 weeks after infection, which were significantly higher than the level in normal, uninfected mice. This period of high TGF-β1 levels in *H. polygyrus*-infected mice corresponded to the time of initiation of coinfection with *P. chabaudi* AS. Our observations suggest that the high level of TGF-β1 induced in *H. polygyrus*-infected mice may suppress immune responses, including the protective IFN-γ-mediated response required for control of acute blood-stage *P. chabaudi* AS infection. The observation that deworming prior to malaria infection restored the ability of *H. polygyrus*-infected mice to control blood-stage malaria in association with a significant reduction in the level of TGF-β1 further supports an important role for this cytokine in nematode-induced immunosuppression. Our results also show that mice infected with blood-stage *P. chabaudi* AS alone produced increased levels of IL-10 in vivo. IL-10 produced during acute malaria has been shown to be important for preventing immunopathology by down-regulating proinflammatory cytokines (25). However, mice coinfected with *H. polygyrus* and *P. chabaudi* AS produced significantly higher levels of IL-10 than mice infected with *P. chabaudi* AS alone. The excessive IL-10 production in coinfected mice may also contribute to the impaired immune protection against blood-stage *P. chabaudi* AS infection. Studies to address the roles of TGF-β1 and IL-10 in suppression of immunity to blood-stage *P. chabaudi* AS and the cell source of these immunoregulatory molecules are currently in progress.

In summary, the results of this study demonstrate that concurrent nematode infection strongly modulates multiple aspects of host immunity to blood-stage malaria, including cytokine production, immune cell function, and antibody response. Consequently, the development of protective immunity to malaria is impaired, leading to significantly increased malaria parasitemia and high mortality.

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