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Immune Responses in Human Necatoriasis: Association between Interleukin-5 Responses and Resistance to Reinfection

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(See the editorial commentary by Maizels and Balic, on pages 427–9.)

Cytokine and proliferative responses to *Necator americanus* infection were measured in a treatment-reinfection study of infected subjects from an area of Papua New Guinea where *N. americanus* is highly endemic. Before treatment, most subjects produced detectable interleukin (IL)-4 (97%), IL-5 (86%), and interferon (IFN)- γ (64%) in response to adult *N. americanus* antigen. Pretreatment IFN- γ responses were negatively associated with hookworm burden, decreasing by 18 pg/mL for each increase of 1000 eggs/gram (epg) ($n = 75$; $P < .01$). Mean IFN- γ responses increased significantly after anthelmintic treatment, from 166 to 322 pg/mL ($n = 42$; $P < .01$). The intensity of reinfection was significantly negatively correlated with pretreatment IL-5 responses, decreasing by 551 epg for each 100 pg/mL increase in production of IL-5 ($n = 51$; $P < .01$). These data indicate that there is a mixed cytokine response in necatoriasis, with worm burden-associated suppression of IFN- γ responses to adult *N. americanus* antigen. Resistance to reinfection is associated with the parasite-specific IL-5 response.

The human hookworms *Necator americanus* and *Ancylostoma duodenale* infect more than a billion people worldwide and are a significant cause of iron-deficiency anemia [1]. The global burden of disease due to hookworms has been estimated to be 22 million disability-adjusted life-years [2]. Infection usually occurs after penetration of the skin by infective larvae, followed by a tissue-migratory larval stage. Adult worms are long-lived inhabitants of the small intestine, and, in contrast to infection with other geohelminths, the intensity of infection is usually greatest in adults [3, 4]. Infection with hookworms is treated easily with a variety of anthelmintics, but, in areas where hookworms are endemic, reinfection after treatment is rapid [5, 6], and

long-term control requires repeated chemotherapy. Recently, attention has been focused on the possibility of control by vaccination, and a number of vaccine candidates are being tested [7]. Relatively little is known about protective immune responses to infection with hookworms. Infection with hookworm, as with other helminths, induces a strong immune response, with elevated levels of total and specific IgE and eosinophilia. There is some evidence of a protective effect of anti-hookworm antibodies [8], and total and specific IgE responses have been shown to correlate negatively with hookworm fecundity [9]. However, the role of cellular and cytokine responses in resistance to reinfection has not been investigated.

Infection with human hookworms is chronic, with adult worms surviving an average of 2–4 years, with a maximum of 18 years [10]. This suggests that infection with hookworms may modulate parasite-specific immune responses. Such immunomodulation is characteristic of infection with tissue-dwelling filarial nematodes and schistosomes, resulting in an antigen-specific suppression of cellular immune responses [11–14]. There

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may also be modulation of responses to nonparasite antigens, such as bacterial antigens [15–17]. Since hookworm-infected communities typically harbor other species of helminth and nonhelminth pathogens, there is the potential for a wide variety of immunological interactions. Such interactions may be of great importance when considering vaccination against either hookworms or other pathogens in areas where hookworms are endemic.

We report here the results of a study of an *N. americanus*-infected population in Papua New Guinea. The aims of the study were to describe the proliferative and cytokine (interferon [IFN]- γ , interleukin [IL]-4, and IL-5) responses to hookworm antigen in an infected population and to test the hypotheses that (1) infection with hookworms suppresses immune responses to parasite or mycobacterial antigen, (2) antihookworm immune responses protect against reinfection, and (3) coinfection with other parasites affects immune responses to hookworm.

SUBJECTS, MATERIALS, AND METHODS

Study population. The study was performed in the village of Haven, Madang Province, Papua New Guinea. Informed consent was obtained from all subjects or their parents. The study was approved by the Medical Research Advisory Committee of Papua New Guinea. Stool samples were obtained during September 1998, and fecal egg counts were performed by use of a modified McMaster salt-flotation method, with results expressed as eggs per gram (epg) of feces. Blood samples (10–20 mL) were obtained from 81 infected subjects >5 years old during November–December 1998 (table 1); those subjects were then treated with a single dose of pyrantel pamoate (10 mg/kg of body weight) 0–3 days after blood samples were obtained. Repeat blood samples were obtained from 54 subjects a median of 33 days after treatment (range, 24–38 days); the remaining subjects refused to have a second blood sample obtained. The efficacy of anthelmintic treatment was assessed in a nearby village: treatment reduced the mean epg by 83% ($n = 44$). At the conclusion of sampling, everyone in the village, except children <4 years old and pregnant women, was offered treatment with a single dose of albendazole (400 mg). Perinatal vaccination with bacille Calmette-Guérin (BCG) is routinely performed in this population. Before treatment, all subjects were tested for malaria infection by use of microscopic examination of Giemsa-stained blood smears and for filarial infection by use of circulating antigen ELISA (TropBio). During September 2001, ~33 months after treatment, reinfection hookworm burden (i.e., epg) was assessed in as many members of the study group as possible ($n = 63$), and treatment with albendazole was offered again. Reinfection epg was not assessed in 14 subjects who were absent from the village and in 4 subjects who did not provide a fecal sample. Blood samples were also

Table 1. Demographic and parasitological characteristics of subjects enrolled in a study of *Necator americanus* infection in Papua New Guinea.

Characteristic	Value
Age, years	
Mean (95% CI)	24.6 (22.0–27.5)
Range	6–66
Sex, no. of subjects	
Male	50
Female	31
Hookworm burden, mean (95% CI), epg	
All ages ($n = 81$)	3182 (2551–3928)
6–13 years old ($n = 13$)	2356 (1041–4095)
14–20 years old ($n = 25$)	3233 (2242–4473)
21–34 years old ($n = 25$)	3742 (2453–5402)
35–66 years old ($n = 18$)	2931 (1870–4524)
Prevalence of other infections, no. of subjects infected/total no. of subjects (%)	
<i>Trichuris trichiura</i>	2/81 (2.5)
<i>Ascaris lumbricoides</i>	0/81 (0)
Filariasis	10/79 (12.7)
<i>Plasmodium</i> species	15/77 (19.5)
<i>P. falciparum</i>	11/77 (14.3)
<i>P. vivax</i>	3/77 (3.9)
<i>P. malariae</i>	1/77 (1.3)

NOTE. CI, confidence interval; epg, eggs per gram of feces.

obtained from 5 control subjects (21–44 years old) from an area where hookworms are not endemic (University of Leeds, Leeds, UK).

Preparation of antigens. Adult *N. americanus* excretory-secretory (ES) products and the mycobacterial antigen purified protein derivative (PPD; Statens Serum Institute, Copenhagen) were used. ES products were obtained as described elsewhere [18]. In brief, *N. americanus* worms were maintained in syngeneic DSN hamsters by percutaneous infection of neonates with 100 infective *N. americanus* larvae. Thirty-five days after infection, the hamsters were killed, and the small intestine was removed, cut along its length, and placed in Hanks' buffered saline solution at 37°C. Adult worms were allowed to detach voluntarily from the small intestine, washed extensively with RPMI 1640 medium supplemented with 100 IU/mL penicillin and 100 μ g/mL streptomycin, and incubated for 1 h at 37°C. ES products were collected by culturing overnight in RPMI 1640 medium supplemented with 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine. The protein content of the collected ES products was determined by use of bovine serum albumin (BSA) standards (BioRad). ES products were freeze-dried and stored at –20°C until required.

Proliferation assays. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by centrifugation over Histopaque 1077 (Sigma). PBMCs were washed twice in wash medium (RPMI 1640 Dutch modification sup-

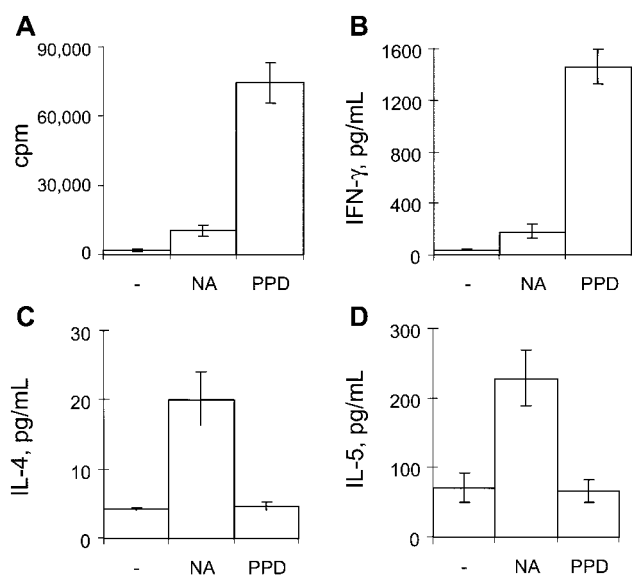


Figure 1. Proliferative and cytokine responses in *Necator americanus*-infected subjects. Mean proliferative (A), interferon (IFN)- γ (B), interleukin (IL)-4 (C), and IL-5 (D) response to medium alone (-), *N. americanus* antigen (NA), and mycobacterial antigen purified protein derivative (PPD). Proliferation is expressed as counts per minute (cpm). Vertical bars, Bootstrap 95% confidence intervals.

plemented with 100 IU/mL penicillin, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 25 μ mol/L 2-mercaptoethanol, counted in 0.4% trypan blue, and resuspended at a concentration of 10^6 cells/mL in wash medium supplemented with 10% fetal calf serum. Cells were cultured in triplicate in 96-well plates, at a concentration of 2×10^5 cells/well, and were incubated at 37°C in 5% CO₂ in the presence of 10 μ g/mL antigen. One hundred microliters of supernatant was removed on day 5, and cells were pulsed with 1 μ Ci/well of tritiated thymidine (DuPont) for 16 h and harvested onto glass-fiber filters. Radioactivity was counted in a scintillation counter (Wallac).

Cytokine determination. Levels of IFN- γ were determined in day-5 supernatants of the cultures used for proliferation assays. For determination of levels of IL-4 and IL-5, parallel cultures were set up at concentrations of 10^7 cells/mL, and supernatants were harvested on days 3 and 5, respectively. Where cells were limiting, cultures for IL-5 determination were not set up. All supernatants were stored at -70°C. Cytokine assays were performed by use of ELISA using commercial kits with a detection limit range of 31–2000 pg/mL for IFN- γ , 7.8–500 pg/mL for IL-5 (Duoset; Genzyme), and 3.9–125 pg/mL for IL-4 (Bio-source). Results were expressed as picograms per milliliter; where levels were above or below the detection limit, a value equal to the detection limit was recorded.

Total IgE ELISA. Polystyrene 96-well plates (MaxiSorp) were coated with 100 μ L of monoclonal mouse anti-human

IgE (clone GE-1; Sigma; 1:500 diluted in 0.05 mol/L carbonate/bicarbonate buffer [pH 9.6]) overnight at 4°C. Plates were washed with PBS plus 0.05% Tween 20 (PBS-T; pH 7.2) and blocked with 200 μ L of 3% BSA in PBS for 1 h at 37°C. After blocking, the plates were washed again, and 100 μ L of human serum (diluted 1:500 in PBS-T plus 1% BSA) was added to each well and incubated for 2 h at 37°C. A standard curve of human IgE standards (doubling dilutions from 125 to 0.5 IU/mL; National Institute of Biological Standards and Control) was included on each plate. All assays were performed in duplicate. Plates were washed again, and 100 μ L of alkaline phosphatase-conjugated goat anti-human IgE (Sigma; diluted 1:1000 in PBS-T plus 1% BSA) was added to each well, and the plates were incubated for 1 h at 37°C. Antibody binding was visualized by the addition of p-nitrophenyl phosphate substrate, and absorbance was measured at 405 nm.

Statistical analysis. Data on proliferation (counts per minute) and production of cytokines were analyzed after subtraction of background (no antigen) levels. Data were analyzed untransformed, with confidence intervals (CIs) of means and regression slopes and significance levels calculated empirically by bootstrapping, using bias correction (2000 replicates). Bootstrapping is a nonparametric method and is appropriate for analyzing highly skewed data, such as cytokine levels, which are not easily transformed to normality [19]. The analysis of variables affecting pretreatment proliferation and cytokine production was by multiple regression. All variables (hookworm epg, presence of filarial infection, presence of malaria, age, age², and sex) were included in the full model, and nonsignificant variables were removed sequentially until only significant variables remained ($P < .05$). Removed variables were then retested in the final model and were retained if significant ($P < .05$). Analysis of variables affecting reinfection hookworm burden was performed by regression, as described above: each immunological variable was analyzed separately, with age, age², sex, and pretreatment hookworm burden included in the full model. All analyses were performed by use of Stata 6.0 software [20]. Since multiple immune response variables were analyzed, most attention was given to results with $P < .01$.

RESULTS

Parasitology. All study subjects were infected with hookworm; *N. americanus* is the only species of hookworm present in the area [4]. The mean intensity of infection with hookworms was 3182 epg (95% CI, 2551–3928 epg). The intensity of infection with hookworms was lowest in children <14 years old (table 1), but there was no significant association between intensity and host age or sex. *Trichuris trichiura* was present at a very low prevalence, whereas *Ascaris lumbricoides* was not detected; *Enterobius vermicularis* infection was not assessed but is known to be present in the study village. The prevalence of

Table 2. Relationship between immune responses to *Necator americanus* antigen (NA) and mycobacterial purified protein derivative (PPD) and parasitic infection and host age and sex, in hookworm-infected subjects in Papua New Guinea.

Parameter, antigen	Hookworm burden, epg	Filariasis positive	<i>Plasmodium</i> positive	Age/age ²	Sex
cpm					
NA	-0.615 (-1.243 to -0.005) ^a	NS	NS	NS	NS
PPD	NS	NS	-26,796 (-45,134 to -8093) ^c	NS	NS
IFN- γ					
NA	-0.0183 (-0.0363 to -0.0068) ^b	NS	NS	NS	NS
PPD	-0.052 (-0.103 to -0.010) ^a	NS	NS	NS	NS
IL-4					
NA	NS	-8.837 (-15.45 to -1.78) ^a	-10.98 (-17.39 to -4.97) ^b	NS	NS
PPD	NS	NS	NS	NS	NS
IL-5					
NA	NS	-125.0 (-199.3 to -32.0) ^a	NS	NS	NS
PPD	NS	NS	NS	NS	NS
Total IgE	0.29 (0.10 to 0.56) ^b	NS	-1507 (-3126 to -232) ^a	NS	-1632 (-2894 to -306) ^a

NOTE. Data are slopes and bootstrap 95% confidence intervals. Proliferative (counts per minute [cpm]) and cytokine responses were analyzed after subtraction of background (no antigen) levels. epg, eggs per gram of feces; IL, interleukin; NS, not significant ($P > .05$; not included in final model).

^a $P < .05$.

^b $P < .01$.

^c $P < .001$.

filial circulating antigen was 12.7%; *Wuchereria bancrofti* is the only species occurring in this region. The prevalence of *Plasmodium* species infection was 19.5%, mostly *P. falciparum* (table 1).

Pretreatment proliferative and cytokine responses. Pretreatment proliferative and cytokine responses are shown in figure 1. Most subjects produced detectable levels of IL-4 (72/74 [97%]), IL-5 (59/69 [86%]), and IFN- γ (47/74 [64%]) in response to *N. americanus* ES antigen. Proliferative and IFN- γ responses to PPD were strong, with all subjects producing detectable IFN- γ (74/74 [100%]). However, few subjects produced detectable IL-4 (8/46 [17%]) or IL-5 (24/54 [44%]) in response to PPD. The mean total IgE level was 3813 IU/mL (95% CI, 3203–4535 IU/mL). In contrast, proliferative and IFN- γ responses to *N. americanus* antigen in control subjects from an area where hookworms are not endemic were below background (no antigen) levels, and there was no detectable IL-4 response.

Immune responses and intensity of infection with hookworms. Both proliferative and IFN- γ responses to hookworm antigen were significantly lower in subjects with high pretreatment hookworm burden (table 2; figure 2A and 2B). This effect was not hookworm specific: IFN- γ responses to PPD were also negatively correlated with hookworm burden (figure 2B). In contrast, IL-4 and IL-5 responses to either antigen were not related to pretreatment hookworm burden. Proliferative and cytokine responses were not significantly correlated with age and sex. Total IgE levels were significantly positively correlated with pretreatment hookworm burden and were lower in female subjects.

Immune responses and coinfection. Proliferative and IFN- γ responses to hookworm antigen were not affected by filarial

or malarial coinfection (table 2). In contrast, proliferative responses to PPD were significantly lower in subjects with *Plasmodium* species infection. Both IL-4 and IL-5 responses to hookworm antigen were significantly lower in subjects with filarial infections (figure 3A and 3B), and IL-4 responses were also lower in subjects with *Plasmodium* species infection (figure 3C). IL-4 and IL-5 responses to PPD were not affected by coinfections. Total IgE levels were lower in subjects with *Plasmodium* species infection.

Effect of anthelmintic treatment. The IFN- γ response to hookworm antigen increased significantly after chemotherapy ($P < .01$), whereas proliferative IL-4 and IL-5 responses to hookworm antigen were unchanged (table 3). In contrast, the proliferative response to PPD decreased significantly after treatment ($P < .001$), as did total IgE ($P < .001$). Variation in the number of days since treatment did not affect the change in most immune responses, although there was a greater decrease in IL-5 *N. americanus* antigen over time, and a lesser decrease in IL-4 PPD over time ($P < .05$ for both).

Immune responses and intensity of reinfection with hookworms. The prevalence of hookworm reinfection after 33 months was 97% (61/63 subjects), and the mean intensity of reinfection was 2144 epg (95% CI, 1498–3046 epg). There was a significant negative relationship between reinfection worm burden and the pretreatment IL-5 response to hookworm antigen ($P < .01$; figure 4). Examination of figure 4 shows 1 point with very high reinfection epg; if this point is removed from the analysis, the slope is -2.64 (95% CI, -5.26 to -0.54; $P < .02$). Reinfection burden was not related to other antihookworm responses, age, sex, or pretreatment hookworm burden.

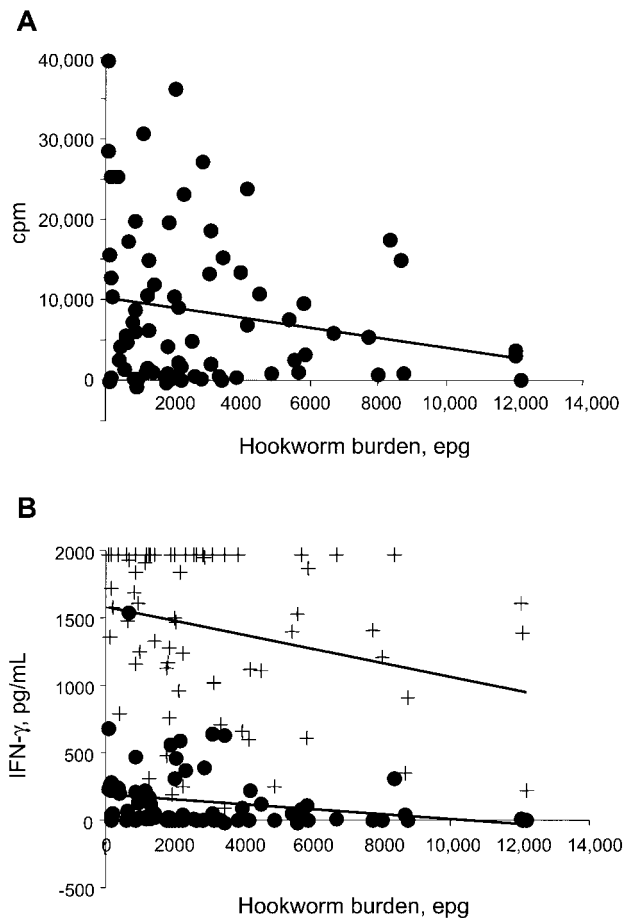


Figure 2. Relationship between *Necator americanus* burden and the proliferative response (expressed as counts per minute [cpm]) to *N. americanus* antigen (A) or the interferon (IFN)- γ response (B) to *N. americanus* antigen (●) and the mycobacterial antigen purified protein derivative (+). Proliferation and IFN- γ responses are values after subtraction of background (no antigen) levels. Worm burden is expressed as no. of eggs per gram (epg) of feces. Linear regression trend lines are plotted.

DISCUSSION

Human immune responses to infection with hookworms, like those to other helminth infections, are characterized by up-regulated production of specific and nonspecific IgE and eosinophilia. We have shown that, as expected, infection with hookworms is associated with up-regulation of the Th2 cytokines controlling these responses, with most infected subjects producing both IL-4 and IL-5 in response to hookworm antigen. However, most subjects also produced detectable IFN- γ in response to hookworm antigen. Adult hookworm antigen was used in the present study, but there is cross-reactivity between larval and adult antigens [21], so some of the observed immune response may have been stimulated by larval stages. Such a mixed Th1/Th2 response to parasite antigens has also

been described in *N. americanus* infection in Africa and in *T. trichiura* infection [22, 23]. In contrast, the immune response to *A. lumbricoides* infection is more Th2 biased, with no detectable production of IFN- γ [24].

There was a clear negative relationship between hookworm burden and IFN- γ responses to hookworm antigen. Such a negative relationship could indicate hookworm-associated immunosuppressive effects or could be evidence of protective immune responses. There was also a significant increase in anti-hookworm IFN- γ responses 5 weeks after chemotherapy. This increase could be due to the removal of immunosuppressive adult worms or to increased exposure to antigens released from dead or dying worms. Since worms are expelled intact from the gut after treatment, the latter possibility is unlikely. Thus, the results of the present study provide strong evidence of an immunosuppressive effect of high hookworm burdens on IFN- γ responses that is removed by chemotherapy. There was also some evidence of an effect on specific proliferative responses, which had a weakly significant negative correlation with hookworm epg and also increased after chemotherapy, although not significantly. The immunosuppressive effect is apparently Th1 specific, because there was no evidence of suppression of IL-4 or IL-5 responses, and total IgE was positively correlated with hookworm burden and decreased significantly after treatment. Changes in IgE may reflect the level of antigenic stimulation and/or down-regulatory IFN- γ responses. Infection with tissue-dwelling filarial nematodes and schistosomes is known to result in the down-regulation of parasite-specific immune responses [11–14], with increased responsiveness after chemotherapy [13, 25, 26]. Immunosuppression has been associated with production of IL-10 and transforming growth factor- β [27–29]. In addition, *N. americanus* produces a range of potentially immunomodulatory molecules, including proteases and a C-type lectin, and adult worm products have been reported to induce apoptosis in activated T cells [30–32]. Whether the specific IFN- γ response protects against infection with hookworms is unknown. However, that hookworms suppress IFN- γ responses suggests that these responses damage adult worms, and some schistosome studies show a protective role for proliferative [26] and IFN- γ [33] responses. Mathematical modeling has shown that the detection of anti-adult worm protective immune responses from immunoepidemiological data can be difficult [34]. In particular, anti-adult worm responses are not expected to be negatively associated with the degree of reinfection [34]; thus, the lack of an association between IFN- γ responses and reinfection seen here does not exclude a protective role for IFN- γ .

The present data show a clear protective effect of IL-5 responses to hookworm antigen. The results were not confounded by associations between reinfection and age, sex, or

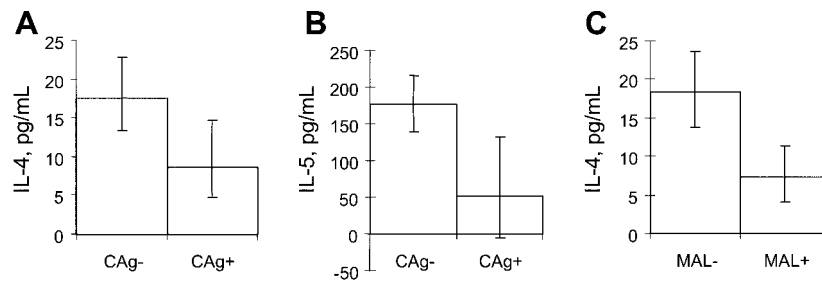


Figure 3. Production of cytokines in response to *Necator americanus* antigen in subjects coinfecting with hookworms and *Wuchereria bancrofti* or *Plasmodium* species. Data are the mean levels of production of interleukin (IL)–4 (A) and IL-5 (B) in subjects positive (+) or negative (–) for circulating filarial antigen (CAg). C, Production of IL-4 in subjects positive or negative for *Plasmodium* species malaria (MAL), as determined by thick smear. IL-4 and IL-5 responses are values after subtraction of background (no antigen) production. Vertical bars, Bootstrap 95% confidence intervals.

pretreatment hookworm burden. This is the first evidence of protective cytokine responses in infection with hookworms. The association with reinfection strongly suggests that IL-5 responses act against incoming larvae, rather than adult worms [34]. Previous studies in Papua New Guinea have shown a negative correlation between antilarval IgG and reinfection [8]. In contrast, epidemiological evidence of immunity is limited: here, neither pretreatment nor reinfection worm burden decreased with increasing host age. However, the relationship between age and exposure is unknown, and comparison across studies does provide evidence of acquired resistance [35]. These studies suggest that Th2 responses are associated with resistance to infection with hookworms. Perhaps surprisingly, there was no association between IL-4 and reinfection, although vaccine-induced immunity in a murine model is associated with increased levels of IL-4 in tissue [36].

The role of IL-5 suggests that eosinophils may be important for protection. This result is also consistent with the observation that *N. americanus* produces metalloproteases that cleave eotaxin and thus inhibit eosinophil recruitment in vitro [37]. Similar studies of schistosome infection have found evidence of a protective role of IL-5 against reinfection [26, 33, 38], although, in one study of *Schistosoma haematobium*, IL-5 was associated with disease, not protection [39]. Th2 cytokines (IL-9, IL-10, and IL-13) have also been associated with low *A. lumbricoides* burden, which is consistent with a protective role, although no associations were seen with *T. trichiura* burden [23, 40]. Our data thus suggest that different immune responses may act against adult and larval hookworms, with strong evidence of IL-5-mediated protection against larvae, and a suggestion of IFN- γ -mediated protection against adult worms, which is suppressed by established adult

Table 3. Effect of anthelmintic treatment on immune responses in *Necator americanus*-infected subjects.

Parameter, antigen	No. of subjects	Pretreatment level, mean	Posttreatment level, mean	Change (bootstrap 95% CI)
cpm				
NA	42	10,339	13,888	+3549 (–175 to 7261)
PPD	42	78,474	59,566	–18,909 (–27,268 to –10,313) ^a
IFN- γ , pg/mL				
NA	42	166	322	+156 (11 to 339) ^b
PPD	42	1436	1463	+27 (–122 to 175)
IL-4, pg/mL				
NA	42	20	17	–3.1 (–7.5 to 1.2)
PPD	29	0.05	–0.1	–0.2 (–1.2 to 0.6)
IL-5, pg/mL				
NA	37	163	151	–12 (–58 to 32)
PPD	23	–11	–0.7	+10 (–29 to 70)
Total IgE, IU/mL	54	4132	3591	–541 (–923 to –254) ^a

NOTE. Proliferative and cytokine responses to *N. americanus* antigen (NA) and mycobacterial purified protein derivative (PPD) and total IgE level, before and after treatment with pyrantel pamoate, and the change (bootstrap 95% confidence interval [CI]) in immune response. Proliferation is expressed as counts per minute (cpm). Proliferative and cytokine responses are values after subtraction of background (no antigen) level.

^a $P < .001$.
^b $P < .01$.

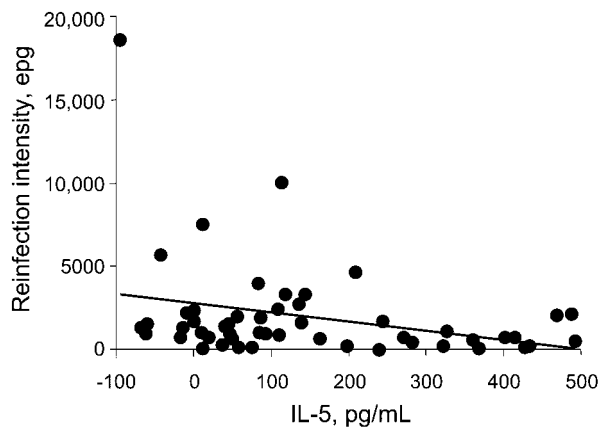


Figure 4. Relationship between the intensity of reinfestation with *Necator americanus* and production of interleukin (IL)-5 in response to *N. americanus* antigen before treatment. Reinfestation was assessed as no. of eggs per gram (epg) of feces 33 months after anthelmintic treatment. IL-5 responses are values after subtraction of background (no antigen) production. Linear regression trend line is plotted; there is a significant negative relationship (slope, -5.512 ; bootstrap 95% confidence interval, -13.376 to -1.266 ; $P < .01$).

worms. There was no evidence of suppression of IL-5 responses by adult worms, perhaps suggesting some concomitant immunity. However, previous studies have shown negative associations between Th2 responses (total and specific IgE) and size and fecundity of adult worms [9]. Although these effects could be mediated by effects on larvae, they suggest some effective anti-adult worm Th2 responses. The present study was a relatively small study of a polyparasitized population, which may have limited our ability to detect any association between adult worms and Th2 responses.

The geographic distribution of infection with hookworms overlaps that of many other parasitic diseases; thus, hookworm-infected subjects will usually be infected with a variety of other parasites. In our study area, both filarial infection and malaria are common, although infections with other gastrointestinal helminths are rare. We have found evidence that both infections suppress Th2 cytokine responses to hookworm antigens. The data suggest that, in polyparasitized subjects, there will be multiple immunological interactions between infections, due to nonspecific immunosuppressive effects and cross-reacting antigens. Active filarial infection was associated with decreased IL-4 and IL-5 responses to hookworm antigens. Although filariasis is typically associated with suppression of IFN- γ responses, there is some evidence of down-regulation of IL-4/IL-5 or IL-5 responses to filarial antigens [41, 42], and proliferative responses to larval hookworm antigen are down-regulated by concomitant *Schistosoma mansoni* infection [43]. Infection with *Plasmodium* species was associated with suppression of IL-4 responses to hookworm antigen. The effects of malaria on helminth infection have not been previously described, although

malaria has been associated with suppression of spontaneous production of IL-4 [44] and a lower risk of atopic skin reactions [45]. The apparent distinction between infection with hookworms, which affected Th1 responses, and filarial or malarial infection, which affected Th2 responses, may reflect the relatively small sample size. Hence, only the strongest influence on each response may have been detected.

There was evidence of a suppressive effect of both hookworm and *Plasmodium* species infection on responses to PPD. IFN- γ responses to PPD were weakly negatively correlated with hookworm burden but did not increase after chemotherapy, in contrast to the results of an Ethiopian study [46]. This suppressive effect on Th1 responses was not associated with a shift toward a Th2 response, because IL-4 and IL-5 responses did not vary. A similar down-regulation of anti-PPD responses has been reported in onchocerciasis [15, 17]. The clinical relevance is unclear, although an association between intestinal nematodes and multibacillary leprosy has been reported elsewhere [47]. It has been suggested that the relatively low efficacy of BCG vaccination in the tropics may result from concomitant helminth infection [48], and ascariasis and onchocerciasis have been reported to reduce post-vaccination immune responses to cholera and tetanus vaccine, respectively [16, 49]. We observed a strong suppressive effect of *Plasmodium* species parasitemia on proliferative responses to PPD. Effects of moderate to severe, but not mild, malaria on PPD responses have been reported elsewhere [50], whereas, in the present study, effects were seen in asymptomatic infected subjects. There was also a surprising decrease in proliferative responses to PPD after chemotherapy. There is no obvious reason why removal of hookworms should suppress anti-PPD responses, and there was no correlation between pretreatment burden and anti-PPD responses. However, because pretreatment anti-PPD responses were strongly affected by *Plasmodium* species infection, it is possible that a change in prevalence of *Plasmodium* species over time may have affected these data. Blood smears were not performed for study subjects at the posttreatment blood sampling; however, smears from other subjects in the village revealed that the prevalence of *Plasmodium* species infection increased from 17.9% during the pretreatment sampling period to 37.7% during posttreatment sampling ($P = .027$, controlling for age and sex). This change in prevalence is likely to have caused the differences in PPD responses and could have obscured other increases in immune responses to both hookworm and PPD.

In summary, our results show that infection with hookworms induces a mixed Th1/Th2 response, with worm burden-dependent suppression of Th1 responses. Despite this immunosuppression, there is evidence of protective immunity against reinfestation, mediated by IL-5. This protective response was

down-regulated by concomitant infection with *W. bancrofti*. These results suggest that a vaccine inducing Th2 responses may be successful. However, immune responsiveness may be compromised by preexisting infection with hookworms or with other parasites.

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