

Immunologic Profiles of Persons Recruited for a Randomized, Placebo-Controlled Clinical Trial of Hookworm Infection

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Abstract. Data from epidemiologic studies suggest that hookworm infections, in establishing an immunologic phenotype conducive to parasite survival, may protect against the development of allergic disease. We describe immunologic findings from a clinical study designed to investigate the safety of iatrogenic hookworm infection in participants with allergic rhinitis. The low, relatively safe level of hookworm infection used in this study was immunogenic, inducing eosinophilia and a significant specific IgG response. Importantly, no potentiation of IgE responses to the environmental allergens to which the participants were sensitized was seen. However, no evidence of systemic immune regulation was seen in infected participants. This finding may indicate that the level of infection or the frequency of infection may have to be altered in future trials to induce a therapeutically conducive immunologic phenotype.

INTRODUCTION

Hookworm infection imparts cost on the human population.^{1,2} Paradoxically, a number of epidemiologic studies of hookworm^{3,4} and other parasitic helminth infections⁵ suggest that infection of tolerable intensity could impart benefit in the form of immunologic modulation of autoimmune and allergic diseases.^{6,7} Additionally, animal models of a variety of human immune-mediated disorders indicate that helminths and/or helminth products moderate pathologic changes. For example, *Schistosoma mansoni* eggs,⁸ *Heligmosomoides polygyrus*,^{9,10} *Trichinella spiralis*,¹¹ and *Hymenolepis diminuta*¹² ameliorate experimental colitis, coincident with suppression of pro-inflammatory cytokines interleukin-12 (IL-12) (p40) and interferon- γ (IFN- γ) and induction of IL-10, IL-4, IL-5, and IL-13. It has been demonstrated that regulatory T cells play a leading role in this helminth-induced moderation of the immune response profile,¹³ although IL-10-producing macrophages¹⁴ and subverted dendritic cells¹⁵ have also been shown to contribute to this moderation.

To explore the costs and benefits of necatoriasis in humans, clinical trials were carried out to assess the effects of the skin to lung to gut migratory pathway of the hookworm, *Necator americanus*, on lung function in participants with allergic rhinoconjunctivitis but not clinically diagnosed asthma. Furthermore, the immunologic profiles of participants undergoing experimental hookworm infection were monitored in parallel to determine whether the low level infection was immunogenic, whether allergic responses in trial participants were altered, and whether any evidence of immune modulation by a relatively small number of parasites could be detected.

We report on this immunologic profiling, and conclude that low-level hookworm infection of tolerable intensity did not potentiate allergic responses to environmental allergens and was significantly immunogenic. However, low-level infection did not induce a significant level of systemically measurable immune regulation, as assessed by number and frequency of natural regulatory T cells and the presence of regulatory cytokines.

MATERIALS AND METHODS

Persons ≥ 18 years of age with symptoms of allergic rhinoconjunctivitis, but no clinically diagnosed asthma or other significant medical disorders, were recruited to the study from February through August 2006. Full details of the inclusion criteria for this study are published elsewhere.¹⁶ Because recruitment and infection coincided with the pollen season for many participants, and because of the known potentiating effect of parasitic helminth infection on allergen IgE responses,^{17,18} pollen data was obtained for locations from which participants were recruited. From a safety point of view, known temporal relationships between allergen exposure and infection would provide valuable data on the potential of hookworm infection to exacerbate allergy to environmental allergens. These data are shown in Figure 1.

In this double-blind study, participants were randomized and administered with either active ($n = 15$) or placebo ($n = 15$) infection, and received 10 *N. americanus* (L₃) larvae in 200 μ L of distilled water or 200 μ L of histamine dihydrochloride solution (1.7 mg/mL), respectively. Treatment was applied to an area of skin on the forearm and covered with gauze and a water-tight adhesive dressing. Larvae were obtained by a culture of fecal material¹⁹ from a healthy human source negative for hepatitis B and C and human immunodeficiency virus. Participants were seen by the clinical researcher weekly for the first 4 weeks and then at 6, 8, 10, and 12 weeks post-treatment, when blood and stool samples were collected and clinical symptoms assessed were as described elsewhere.¹⁶ Full blood counts were carried out and immunologic analyses performed as described below. Fecal egg content was quantified using a salt flotation technique.³ During the screening visit and at week 12 post-treatment, skin sensitivity towards grass pollen, dust mite allergen, and cat fur was assessed by skin prick testing.¹⁶ Upon conclusion of the study, hookworm-infected participants were provided with a standard course of mebendazole (Vermox; Janssen-Cilag Ltd., High Wycombe, United Kingdom) to eradicate the infection.

The study was reviewed and approved by the Nottingham Research Ethics Committee and Research and Development at the Nottingham University Hospitals National Health Service Trust. All participants were fully informed and written consent was obtained before participation in the study.

Serologic analyses. Plasma was separated from heparinized whole blood by centrifugation at $710 \times g$ for 10 minutes. Total

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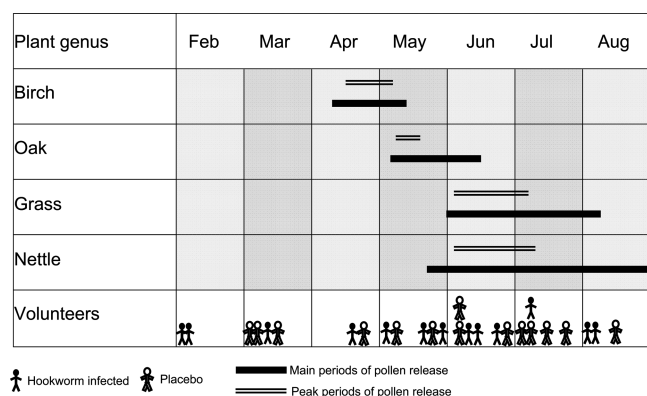


FIGURE 1. Timing of recruitment of study participants relative to peak pollen seasons for the four major pollen types in the Midlands, United Kingdom, summer, 2006. Eight of the study participants did not reside in the Midlands region. Analyses of pollen data for these additional areas did not show any major differences in terms of the pollen types assessed. Pollen release data were provided by National Pollen and Aerobiology Research Unit, University of Worcester.

IgE and parasite-specific IgE and IgG were measured using an enzyme-linked immunosorbent assay optimized in our laboratory.²⁰ Polystyrene 96-well plates were coated with mouse anti-human IgE (total IgE; clone G7-18; BD Pharmingen, San Jose, CA) or adult *N. americanus* excretory/secretory (ES) products (specific IgE/IgG) and incubated with plasma samples. Bound antibody was detected using biotinylated mouse anti-human IgE (clone G7-16; BD Pharmingen) and horseradish peroxidase (HRP)-conjugated streptavidin or HRP-conjugated anti-human IgG (Binding Site, Birmingham, United Kingdom). Plates were developed through the addition of 3,3',5,5'-tetramethylbenzidine containing 0.02% hydrogen peroxide and the reaction was stopped using 2.5 M sulfuric acid. Absorbances at 450 nm were measured and compared with a standard curve of known antibody concentrations on each plate.

Antigen recognition by post-infection plasma IgG was analyzed by Western blot. Briefly, adult *N. americanus* ES products were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membrane. After incubation with noninfected or post-infection plasma, bound IgG was visualized using sheep anti-human IgG-HRP (Binding Site) and 4-chloro-1-naphthol substrate containing 0.02% hydrogen peroxide. At weeks 0, 4, 8, and 12, specific IgE for the allergens mixed grass pollen, dust mite, and cat fur was measured using the CAP-fluorescent enzyme immunoassay system (Phadia, Uppsala, Sweden).

Phenotypic analyses and cytokine release profiles of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by buoyant density gradient centrifugation (Histopaque-1077; Sigma, St. Louis, MO) at $600 \times g$ for 20 minutes. Harvested PBMC were washed twice with RPMI 1640 medium (Sigma) and resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 100 IU/mL of penicillin/100 µg/mL of streptomycin (CTCM). Isolated PBMCs were cultured in 96-well round-bottomed culture plates (2×10^5 cells/well) with 1 µg/mL of *Staphylococcus* enterotoxin B (SEB) or CTCM

alone for 4 days at 37°C in a humidified 5% CO₂ environment. Culture supernatants were collected and stored at –40°C before analysis. Duplicate cell culture supernatants were screened for the release of 13 cytokines (granulocyte–monocyte colony-stimulating factor, IFN-γ, IL-10, IL-12 [p70], IL-13, IL-17, IL-1a, IL-2, IL-4, IL-5, IL-6, human interferon-inducible protein 10, and tumor necrosis factor-α) and 1 chemokine (eotaxin) using the xMAP multi-analyte fluorescent bead profiling technology. Cytokines/chemokine were quantified with the Beadlyte human 14-plex multi-analyte detection system (Upstate/Millipore, Billerica, MA), and determined by a Luminex 100 system (Luminex Corporation, Austin, TX).

To identify T cell subsets, potassium EDTA-treated whole blood samples were labeled using Cyto-stat TetraCHROME™ (Beckman Coulter, Fullerton, CA) monoclonal antibody mixtures (CD45, CD4, CD8, CD3 and CD45, CD56, CD18, and CD3) and ImmunoPrep™ reagents (Beckman Coulter). To enable quantification, Flow-Count™ Fluorospheres (Beckman Coulter) were added to samples. Data were collected using an EPICS XL flow cytometer (Beckman Coulter) to determine absolute numbers of T cells, natural killer cells, and B cells. To identify peripheral blood regulatory T cells, isolated PBMCs were labeled with fluorochrome-conjugated antibodies against CD3 (Beckman Coulter), CD4, CD25, and Foxp3 using a regulatory T cell staining kit (Biolegend, San Diego, CA). Samples were analyzed using an Altra flow cytometer (Beckman Coulter). Data were analyzed using WEASEL version 2 software (The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia).

Statistical analyses. All data were analyzed using Prism version 4.0 (Graphpad Software Inc., San Diego, CA). Because each variable was measured at multiple time points, we computed area under the curve as a summary measure, consistent with the procedure of Feary and others¹⁶ and compared between the two experimental groups using independent samples *t*-test or a non-parametric equivalent if not normally distributed.

RESULTS

Three participants withdrew from the study. One of these withdrew from the placebo group on day 6 because of an intercurrent viral illness, and two participants withdrew from the hookworm group; the first on day 12 because of pregnancy, and the second on day 40 because of abdominal pain and diarrhea. Only those participants who completed the study are shown in Figure 1 and included in the statistical analyses. In addition to the results discussed below, there were no changes identified in the propensity of SEB-stimulated PBMCs to produce any of the other cytokines or chemokines assayed within this study.

Immunogenicity of low-level hookworm infection. Justification of the therapeutic use *N. americanus* infection necessitates the generation of an immunologic response in the absence of adverse effects. We have reported previously²¹ and elsewhere for this study¹⁶ that infection was generally well tolerated with no significant clinical side effects observed.

Anemia is a significant consequence of field infections of *N. americanus*.²² Complete blood counts indicated that hematologic parameters for all participants were within the reference range on entry into the study. There were no significant changes in hemoglobin levels or hematocrit observed in any of the study participants (Figure 2A). Evidence that hook-

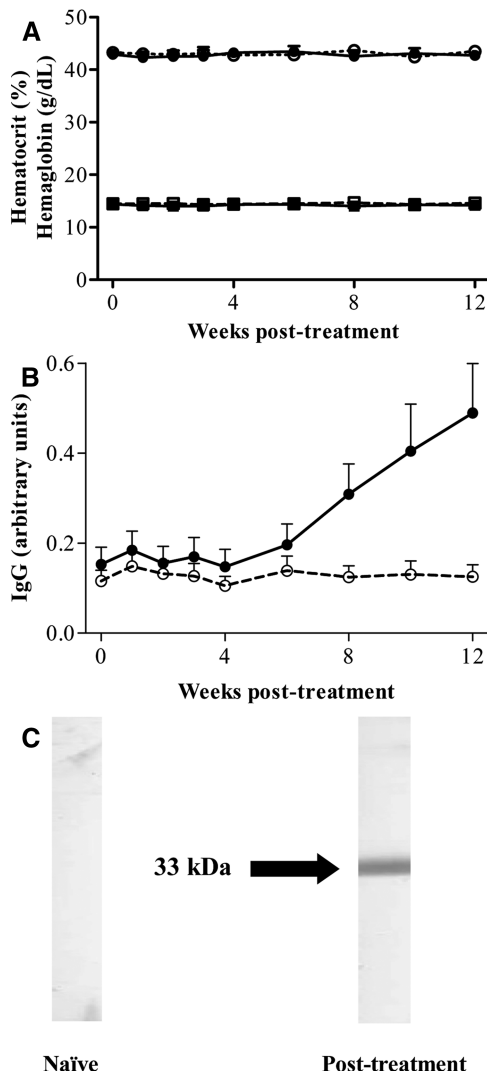


FIGURE 2. Quantification of anemia in study participants by peripheral blood hematocrit (hookworm infected [•], placebo [○]) and hemoglobin (hookworm infected [■], placebo [□]) (A). Infected participants (•) had significantly ($P < 0.05$) increased parasite-specific IgG within peripheral blood plasma as measured by enzyme-linked immunosorbent assay (B) and a characteristic *Necator americanus* Western blot profile (C). Values are the mean + SEM. Multiple time point data was assessed by area under the curve analysis and significant differences were identified using the independent samples *t*-test.

worm infection was recognized immunologically was provided by a significantly heightened parasite-specific IgG response detected by enzyme-linked immunosorbent assay in plasma of infected participants notable at weeks 8, 10, and 12 (Figure 2B). Detection of hookworm-specific IgG was confirmed by a characteristic *N. americanus* Western blot profile²³ (Figure 2C).

Parasite induced eosinophilia without potentiation of anti-allergen IgE. Participants in the hookworm group exhibited peripheral blood eosinophilia, which peaked at approximately six weeks post-treatment (Figure 3A). In relation to this finding, PBMCs from infected participants stimulated with the T cell superantigen, SEB, secreted higher levels of IL-13 at week 3 (Figure 3B), and IL-5 at week 4 (Figure 3C), although these findings were not significant when assessed by area under the curve analyses.

Because it has been reported that parasitic infection may non-specifically potentiate unrelated IgE antibody responses,^{17,18} we used a highly accurate automated system to monitor levels of specific IgE towards the allergens used in the skin prick testing (grass pollen, dust mite, and cat fur) to which the participants were sensitive.¹⁶ There were no changes in the plasma levels of IgE against any of the allergens measured in either the placebo or the hookworm-infected group. Grass pollen-specific IgE data is shown as a representative example (Figure 3D). There were no significant changes in either total (Figure 3E) or parasite-specific IgE over the period of the study. Similarly, there were no changes in the propensity of isolated SEB-stimulated PBMCs to produce IL-4 (Figure 3E).

Absence of evidence for systemic immune regulation after low-level hookworm infection. Eggs were detected in fecal samples of nine of the participants in the hookworm group at 6–8 weeks post-treatment (Figure 4A). Three of the remaining four participants from the hookworm-infected group showed increased eosinophil counts, which suggested that infections had occurred in these persons. In SEB-stimulated PBMC cultures from the hookworm-infected group at week 6, there were no significant changes in production of tumor necrosis factor- α (Figure 4B), but there was a noticeable, but not statistically significant, decrease in production of IFN- γ (Figure 4C). These findings coincided with initial detection of eggs in feces. However, there was no significant change in IL-10 production (Figure 4D), and analysis of peripheral blood T cell populations by flow cytometry showed no change in the number of T cells (Figure 4E) or in the relative percentage or absolute counts (Figure 4F) of regulatory T cells, as identified by expression of Foxp3 in the CD25hi population of CD4 T cells.

DISCUSSION

We have shown that low-level hookworm infection is immunogenic, and the immunologic profile shows some of the characteristics of natural infection with *N. americanus*.^{3,24,25} This finding is important if iatrogenic infection is ever to mimic the reported benefits of natural infection. Although hookworm infections in the field are usually associated with increased parasite specific IgE,²⁰ our data are consistent with those of other publications, in which multiple exposure to iatrogenic infection seems to be required to induce serologically detectable anti-hookworm IgE.^{26–29} Our results show that iatrogenic infection did not potentiate IgE responses against the allergens to which participants were sensitized, which is important for future trial safety, given that infection was superimposed on active anti-allergen IgE responses, conditions that are most conducive to the exacerbation of these responses. However, it may also be true that the level of infection used was too low for potentiation, although it was clearly immunogenic as seen from the eosinophilia observed and the non-significant, but chronologically compelling, increase in IL-5 and IL-13 from *ex vivo*-stimulated PBMCs.

The peak in IL-5 and IL-13 responses and the accompanying eosinophilia are not maintained, and the reasons for this can be discussed. For example, the transient, albeit non-significant, increase in IL-5 and IL-13 production may be related to the passage of larvae through the lung. Previous studies³⁰ showed the importance of larval stages, IL-5, and eotaxin in driving

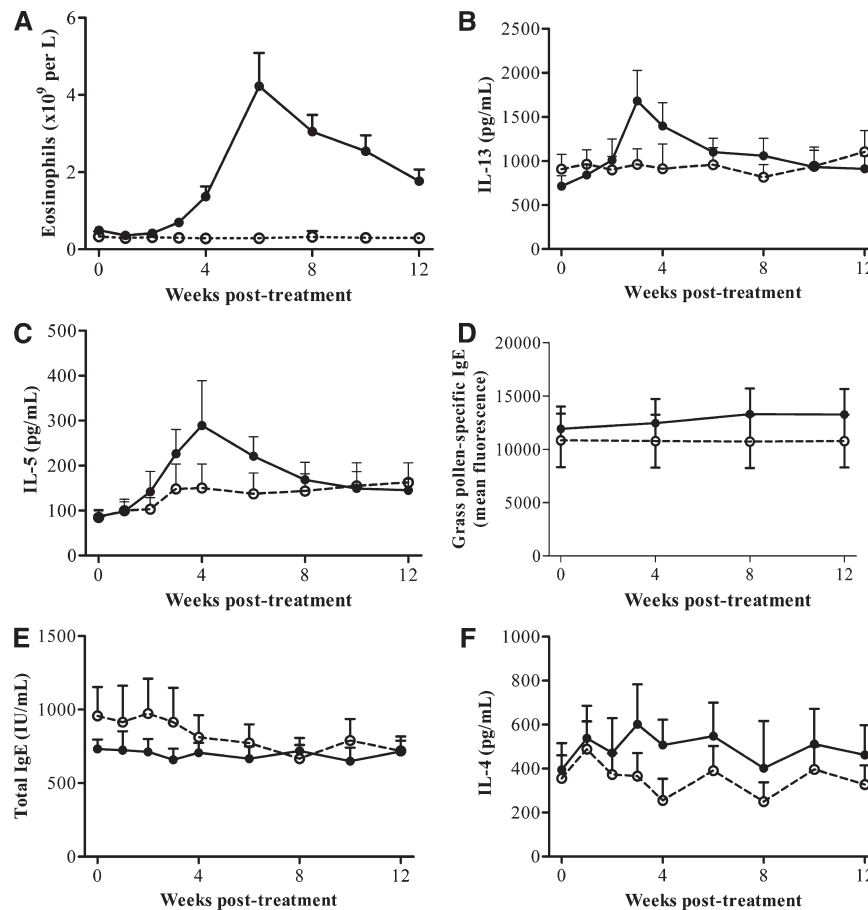


FIGURE 3. Peripheral blood eosinophilia ($P < 0.05$) in hookworm-infected participants (A). Peaks in *Staphylococcus* enterotoxin B-induced release of interleukin-13 (IL-13) (B) and IL-5 (C) from peripheral blood mononuclear cells were observed although these were not significant. There was no potentiation of grass pollen-specific IgE in infected participants (D) and no differences in total IgE (E) or in IL-4-producing T cells (F) between hookworm infected and placebo-treated participants. Values are the mean + SEM. Multiple time point data were assessed by area under the curve analyses and significant differences were identified using the independent samples *t*-test. ● = hookworm infected; ○ = placebo.

this response. Alternatively, the return to background levels could be indicative of down-regulation or active suppression of the Th2 response as the adult mature in the gut. Further work investigating the local immune environment in infected hosts would be required to explain this phenomenon.

Nonetheless, for iatrogenic hookworm infection to be immunologically beneficial, it would need to moderate the immune system to suppress immune responses causing immunologic disease. The level of infection administered in the present study, although relatively safe, did not induce statistically significant levels of systemic immune modulation in the host, as assessed by PBMC responses to the T cell superantigen, SEB, and regulatory T cell frequency.

In summary, infection with *N. americanus* of tolerable intensity, induced a natural immune phenotype³¹ in participants, and did not boost existing allergic responses to environmental allergens. However, no clear evidence of immune regulation was observed. The timing of infection in the current trial was not ideal because most participants were recruited during the hay fever season, but the tolerability of infection indicates that the infection protocol could be suitably modified to determine conclusively whether hookworm infection will be of benefit to patients with allergy. Parallel experiments in animal model systems, using significantly higher worm burdens in terms of

body mass ratio are reporting therapeutic benefit in association with the activation of regulatory networks.^{9–11} Clearly, our past work has shown that worm burdens of this relative magnitude are clinically unacceptable to humans,²² but nevertheless, the current report describes another step in an immunologic journey to find the correct balance between safety and potential therapeutic benefit.

To date, we have shown that iatrogenic hookworm infection caused no significant reduction in lung function in hay fever patients in the presence of clinically insignificant adverse effects,¹⁶ and, herein, did not potentiate allergen-specific IgE, and induced a natural immune phenotype. Trials in the future may necessitate repeated low-level infection at appropriate time periods in an attempt to give rise to the immune suppressive phenotype that may be required to impart therapeutic benefit. For example, larger scale or repeated low level infection before the onset of the hay fever season may be conducive to therapeutic benefit because the regulatory arm of the immune system may be primed before exposure to allergen. Additionally, because *N. americanus* is long-lived within its human host, immune modulation may occur over time, which suggests that monitoring of participants over longer periods may show more striking changes in immune status. Locally occurring immune suppressive events may also be conducive

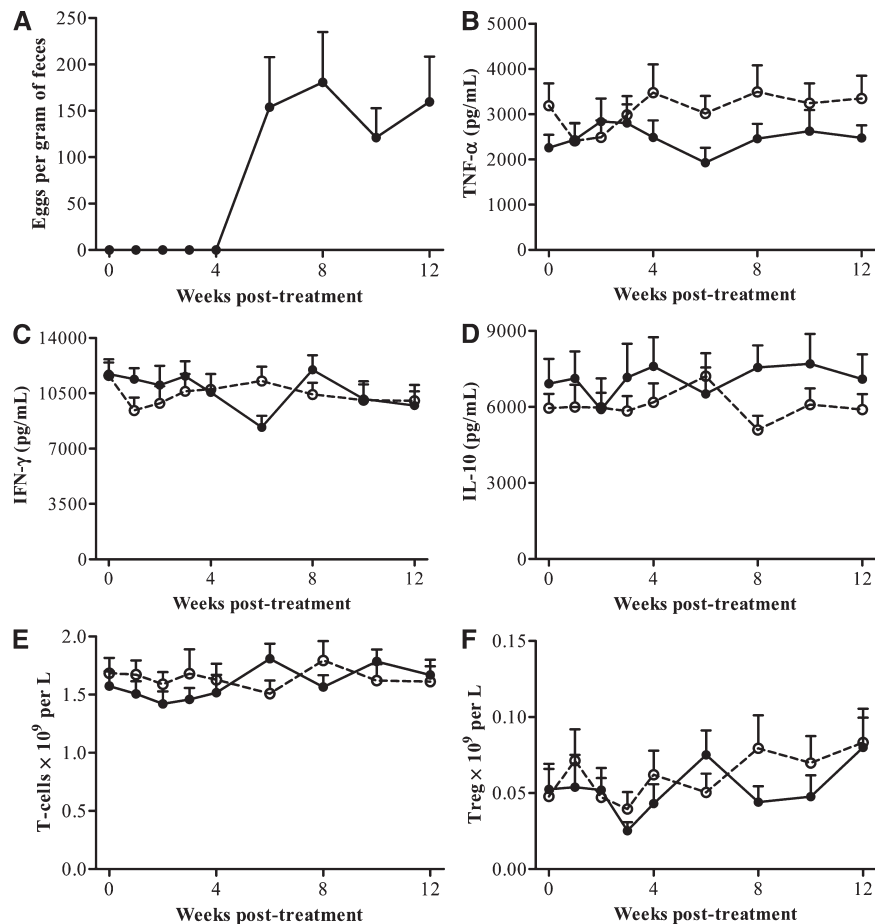


FIGURE 4. Identification of eggs in the fecal samples of infected participants for confirmation of the presence of adult hookworm in the gut (A). There were no significant changes to tumor necrosis factor- α (B), a non-significant reduction in levels of interferon- γ (C), and no change in interleukin-10 (D) produced by *Staphylococcus* enterotoxin B-stimulated peripheral blood mononuclear cells from hookworm-infected versus placebo-treated study participants. No significant differences were observed in absolute counts of peripheral blood T cells (E) or regulatory T cells (F). Values are the mean $\pm$ SEM. Multiple time point data were assessed by area under the curve analyses and significant differences were identified using independent samples *t*-test. $\bullet$ = hookworm infected; $\circ$ = placebo.

to therapeutic benefit, for example in the lung and gut. Thus, we believe significant new knowledge had been gained in this field, which will be of interest to patients, their clinicians, and scientists in general.

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