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# Comparison of Cytokine Responses in Ecuadorian Children Infected with *Giardia*, *Ascaris*, or Both Parasites

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*Abstract.* More than 2 billion people are infected with parasites globally, and the majority have coinfections. Intestinal protozoa and helminths induce polarizing $CD4^+$ T-helper cell 1 (Th1) mediated cytokine responses within the host. Such immune polarization may inhibit the ability of the host to mount an adequate immune response for pathogen clearance to concurrent pathogens. The current study evaluated the plasma cytokine profile in *Ascaris* and *Giardia* coinfected children compared with *Giardia*- and *Ascaris*-only infected children. Fecal samples and blood samples were collected from asymptomatic 3-year-old children living in the district of Quininde, Ecuador. Stool samples that tested positive for *Giardia lamblia*-only, *Ascaris lumbricoides*-only, or *G. lamblia* and *A. lumbricoides* coinfections were confirmed by quantitative real-time polymerase chain reaction. Plasma samples from the study subjects were used to quantitate cytokines. A total of 39 patients were evaluated. Children with coinfection had a significant decrease in Th1 cytokine production, interleukin 2 (IL-2) ($P < 0.05$), IL-12 ($P < 0.05$), and tumor necrosis factor alpha ($P < 0.05$) compared with *Giardia*-only infected children. Coinfected children had an increase in IL-10/interferon gamma (IFN-γ) ratio compared with uninfected ($P < 0.05$) and *Ascaris* alone ($P < 0.05$). The increased IL-10/IFN-γ ratio in the setting of decreased Th1 cytokine response indicates Th2 polarization in the coinfected group. Reduced Th1 cytokines in children coinfected with *Ascaris* and *Giardia* may impair the host's ability to eradicate *Giardia* infection leading to chronic giardiasis.

## INTRODUCTION

More than 2 billion people are infected with parasitic infections worldwide and, not uncommonly, many have coinfections with more than one parasite.[1–3] Coinfections with soil-transmitted helminths (STHs) and protozoal parasites are particularly prevalent in young children.[4] However, until recently, diagnosis of coinfection has been limited by the poor sensitivity of conventional microscopic diagnostic techniques.[5] As diagnostic technologies have evolved with improved throughput and increased sensitivity, the ability to detect coinfections within an individual host has become more feasible.[5] Multiparallel quantitative real-time polymerase chain reaction (qPCR) has allowed for concurrent detection of not only STHs, including *Ascaris lumbricoides*, but also intestinal protozoa, such as *Giardia lamblia*.[4–6] Coinfection specifically with *A. lumbricoides* and *G. lamblia* is common due to overlapping geographic prevalence and similar transmission dynamics in resource-limited areas.[6] We have observed within a birth cohort in rural Ecuador that 213 children at 3 years of age were 16.4% infected with *A. lumbricoides* and 52.1% infected with *G. lamblia.* Of those with ascariasis, 77.4% were coinfected with *G. lamblia* while 34.3% were only infected with *G. lamblia* (R. Mejia, unpublished data), raising the statistical possibility that the immunomodulatory effects of ascariasis may play a role in host susceptibility to giardiasis.[7] Alternative explanations could include changes in the host intestinal environment as a result of mucosal integrity breakdown or shifts in the commensal microbiome allowing for secondary infection.

Ascariasis and giardiasis cause significant chronic morbidity in children.[7] Ascariasis, although often asymptomatic in lightly infected children, is a common cause of abdominal pain in endemic areas and occasionally causes intestinal and biliary obstruction.[4,7] Chronic infections can lead to significant malabsorption, nutrient deficiencies, growth delays, and cognitive impairment.[4,7] Helminth infections such as ascariasis have also been associated with increased susceptibility to malaria, tuberculosis, and human immunodeficiency virus/acquired immunodeficiency syndrome.[8–10] Giardiasis causes acute symptoms of diarrhea, abdominal pain, flatulence, and nausea. In over 50% of infected individuals the illness is self-limiting.[11–13] However, chronic, persistent infection with *Giardia* can occur and lead to chronic wasting syndrome or failure to thrive.[13,14] Persistent giardiasis has been most commonly described in children with altered immune responses, including hypogammaglobulinemia, common variable acquired immunodeficiency, nephrotic syndrome, and protein-calorie malnutrition.[12,15,16]

The interaction between the host immune response and STH and protozoal coinfections is complex involving both the innate and acquired immune responses.[3] Protozoa stimulate a predominantly $CD4^+$ T-helper cell 1 (Th1) mediated cytokine profile. Th1 pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin 2 (IL-2), and IL-12, may play an important role in the elimination of protozoa from the human host.[3,13,17] Previous studies have shown that $CD4^+$ T cells and certain Th1 cytokines, specifically TNF-α and IFN-γ, determine parasite load and parasite clearance, whereas the absence of such an immunologic response contributes to the establishment of chronic giardiasis.[18] Conversely, STHs such as *A. lumbricoides* stimulate a polarized Th2-mediated cytokine profile.[19] Th2 anti-inflammatory cytokines, IL-4, IL-5, and IL-13, promote the activation of mast cells, eosinophils, basophils, and B-cell-mediated immunoglobulin and IgG4 production. Ascariasis has also been associated with expansion of monocytes and regulatory T cells (Tregs) leading to increased levels of the regulatory cytokines, transforming

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growth factor (TGF-β) and IL-10.[8,9] Immunomodulation through upregulation of IL-10 and TGF-β allows for persistence of helminth infection.[8,17,20] Murine studies suggest that helminths induce a profound Th2 response at the expense of the Th1 response leading to downregulation of serum pro-inflammatory cytokines.[8,9,20,21] Th2 polarization and Th1 downregulation during helminth infection may protect the host from significant tissue pathology but may reduce the host capacity to respond effectively to infections with intracellular organisms.[8,9,20,21]

We hypothesized that children coinfected with the STH infection, *A. lumbricoides*, and the intestinal protozoa, *G. lamblia*, have increased immune regulation associated with a reduction in Th1 plasma cytokine concentrations compared with children infected with either parasite only, allowing for persistent giardiasis.

## METHODS

**Sample and data collection and study design.** This was a cross-sectional analysis of 39 asymptomatic 3-year-old children analyzed within an Ecuadorian birth cohort, the Ecuador Life cohort of children living in the district of Quininde, Esmeraldas Province, Ecuador.[22] Participants were selected from an initial random subsample of 400 children providing stool samples at 13 months of age,[5] of whom 213 provided a stool sample at 3 years of age. Blood samples for plasma were collected at 3 years of age. The final sample for analysis of 39 children selected based on having a plasma sample for analysis and the presence of DNA in stool for *G. lamblia*, *A. lumbricoides*, both parasites, and absence of any other common helminth (*Ancylostoma duodenale*, *Necator americanus*, *Strongyloides stercoralis*, and *Trichuris trichiura*) or protozoal infection (*Cryptosporidium hominis/parvum* and *Entamoeba histolytica*)[23] was as follows: 11 patients with *Giardia* only, seven children with *Ascaris* only, and 13 children with *Ascaris* and *Giardia* coinfection. Additionally, eight children, negative for all parasites by qPCR, were used as an uninfected control group. Stools were also examined using a combination of microscopic methods including direct saline mounts, Kato–Katz, and formol-ethyl acetate concentration methods at the time of collection for helminth and protozoal parasites. Results of stool microscopy and appropriate antiparasite medicines were given to the guardian of each child. Aliquots of stool and plasma samples were frozen without fixatives at –80°C until analysis. Data on baseline characteristics were collected using a questionnaire administered in Spanish to the child's primary guardian around the time of birth of the child.[22]

**DNA extraction and multiparallel qPCR.** DNA was extracted from 50 mg stool using the FastPrep® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA),[6] according to the manufacturer's instructions for all parasites except *T. trichiura*. An additional step required for the extraction of *T. trichiura* DNA was performed as previously described.[5] Multiparallel qPCR was performed as previously described[5] with the species-specific primers to identify eight gastrointestinal parasite pathogens including *A. lumbricoides*, *A. duodenale*, *N. americanus*, *S. stercoralis*, *T. trichiura*, *C. parvum*, *E. histolytica*, and *G. lamblia*. Samples that tested positive by qPCR for *G. lamblia, A. lumbricoides*, and *G. lamblia* plus *A. lumbricoides* were used in this study.

**Cytokine assay.** Plasma collected from the study subjects at 3 years of age were used to quantitate cytokines using Bio-Rad Luminex Magpix® (Hercules, CA). Bio-Plex Pro™ Human Cytokine Th1/Th2 Assay (Bio-Rad) were used per the manufacturer's protocol to detect Th1 and Th2 dominant cytokines including IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, granulocyte macrophage colony stimulating factor, TNF-α, and IFN-γ. Cytokine values below the detection limit of the assays were assigned a nominal value of 0 pg/mL. Standard curves for each cytokine were measured along with the samples.

**High-sensitivity C-reactive protein assay.** Levels of C-reactive protein (CRP), a biomarker of systemic inflammation, were measured in plasma samples from study children. High-sensitivity C-reactive protein (hsCRP) ELISA (MP Biomedicals) was used according to the manufacturer's instructions.

**Statistical analysis.** Sample size was based on cost considerations. The primary analysis was comparisons in levels of Th1, Th2, T regulatory (i.e., IL-10), and IL-10/IFN-g ratios between coinfected and singly infected children with the uninfected group included as the control. The Kruskal–Wallis test was used to compare the four groups with two-group analyses performed using the Mann–Whitney test. Statistical analyses were performed using Prism v 5.0 d (GraphPad, La Jolla, CA). *P* values less than 0.05 were considered significant.

**Ethical approval and consent.** Informed written consent was obtained from each participant or from a parent/guardian. Antiparasitic treatment, based on microscopy findings, was provided per standard of care in the region. The subjects were preschool children and did not receive school-based mass drug administration with anthelmintics. Study protocols were approved by the bioethics committee of Hospital Pedro Vicente Maldonado, Pichincha Province, and by the institutional review board of Baylor College of Medicine.

## RESULTS

Baseline characteristics of the children in the four infection groups are shown in Table 1. The distributions of characteristics between the four groups were comparable and none showed significant intergroup heterogeneity. Coinfection with *A. lumbricoides* and *G. lamblia* was found to be associated with reduced Th1 cytokine production, specifically TNF-α ($P = 0.0148$), when the groups were compared. Further subset analysis of *A. lumbricoides* and *G. lamblia* coinfected group compared with *G. lamblia*-only infected children demonstrated reduction in IL-2 (3.834 versus 8.082 pg/mL; $P < 0.05$), IL-12 (4.719 versus 14.89 pg/mL; $P < 0.05$), and TNF-α (1.518 versus 2.579 pg/mL; $P < 0.05$) (Figure 1). *Ascaris lumbricoides* and *G. lamblia* coinfection was not associated with an increase in serum Th2-mediated cytokines including IL-4, IL-5, or IL-13 compared with participants with monoparasitic infection (Figure 2). In addition, there was no significant difference in immune regulatory IL-10 level between *A. lumbricoides* and *G. lamblia* coinfected children and *A. lumbricoides*-only infection, and *G. lamblia*-only infection (Figure 3).

However, there was a significant difference in the IL-10/IFN-γ ratio between the groups (P = 0.0136). In post hoc subset analysis, the *A. lumbricoides* and *G. lamblia* coinfected children were found to have a significant increase

TABLE 1
Baseline characteristics of 39 children aged 3 years by infection group

| Characteristic | Uninfected ($n$ = 8) | *Giardia* only ($n$ = 11) | *Ascaris* only ($n$ = 7) | *Ascaris* and *Giardia* ($n$ = 13) | $P$ value |
|---|---|---|---|---|---|
| Sex (%) | | | | | |
| Male | 37.5 | 36.4 | 28.6 | 61.5 | 0.440 |
| Female | 62.5 | 63.6 | 71.4 | 38.5 | |
| Nutritional status, mean (SD) | | | | | |
| Weight (kg) | 16.3 (3.1) | 16.8 (3.6) | 16.7 (2.5) | 16.6 (1.1) | 0.983 |
| Height (cm) | 105.3 (7.3) | 104.8 (6.5) | 106.2 (4.7) | 106.6 (4.3) | 0.885 |
| SES (%) | | | | | |
| Low | 25.0 | 27.3 | 28.6 | 30.8 | |
| Medium | 25.0 | 54.6 | 57.1 | 23.1 | |
| High | 50.0 | 18.2 | 14.3 | 46.2 | 0.488 |
| Residence (%) | | | | | |
| Urban | 87.5 | 81.8 | 71.4 | 69.2 | |
| Rural | 12.5 | 18.2 | 28.6 | 30.8 | 0.752 |
| Overcrowding (%) | | | | | |
| No | 50.0 | 54.6 | 42.9 | 46.2 | |
| Yes | 50.0 | 45.4 | 57.1 | 53.9 | 0.963 |
| Birth order, mean (SD) | 2.6 (1.9) | 3.6 (1.8) | 3.0 (1.6) | 2.5 (1.8) | 0.470 |
| Maternal factors | | | | | |
| Educational status (%) | | | | | |
| Illiterate | 12.5 | 9.1 | 14.3 | 30.8 | |
| Complete primary | 62.5 | 63.6 | 71.4 | 46.2 | |
| Complete secondary | 25 | 27.3 | 14.3 | 23.1 | 0.835 |
| Maternal ethnicity (%) | | | | | |
| Afro-Ecuadorian | 37.5 | 9.1 | 42.9 | 30.8 | |
| Other | 62.5 | 90.9 | 57.1 | 69.2 | 0.375 |

SD = standard deviation; SES = socioeconomic status. Household overcrowding defined by ≥ 3 persons per sleeping room. $P$ values represent comparisons between the three infection groups using the Kruskal–Wallis or $\chi^2$ tests as appropriate.

in IL-10/IFN-γ ratio compared with children with *A. lumbricoides*-only infection (0.4761 and 0.1724 pg/mL; $P < 0.05$) (Figure 4). There was no significant difference in *A. lumbricoides* DNA levels signifying burden of disease between coinfected and *A. lumbricoides*-only infected children (26.21 versus 29.94 fg/μL).

There was no significant difference of systemic inflammation across the groups by measurement of plasma hsCRP (*G. lamblia*–only, 1.62 mg/L; *A. lumbricoides*-only, 1.53 mg/L; and *A. lumbricoides* and *G. lamblia* coinfections, 3.28 mg/L; $P = 0.5283$, graph not shown). Mean levels of hsCRP in the uninfected group were 3.06 mg/L.

## DISCUSSION

Parasitic infections, including STHs such as *A. lumbricoides* and intestinal protozoa such as *G. lamblia*, are a major cause of morbidity worldwide particularly in children.[24] Because of the epidemiologic overlap and the complex interactions of helminths and protozoa with the host immune system, children in endemic regions commonly have coinfections with *Ascaris* and *Giardia*.[4,7] Coinfections with multiple parasites are associated with reduced treatment efficacy, increased treatment costs, and overall worse health outcomes.[25]

In this study, we compared serum Th1 and Th2 cytokine production in four groups of asymptomatic children living in an *Ascaris*- and *Giardia*-endemic area of Ecuador: uninfected, *Ascaris*-only infected, *Giardia*-only infected, and *Ascaris* and *Giardia* coinfected children. Children coinfected with *Ascaris* and *Giardia* were noted to have decreased serum Th1 cytokine concentrations compared with children with *Giardia*-only infection. Th1 cytokines are necessary for expansion of humoral and cellular effector cells responsible for *Giardia* clearance.[13,16] Reduction of plasma Th1 cytokines as a result

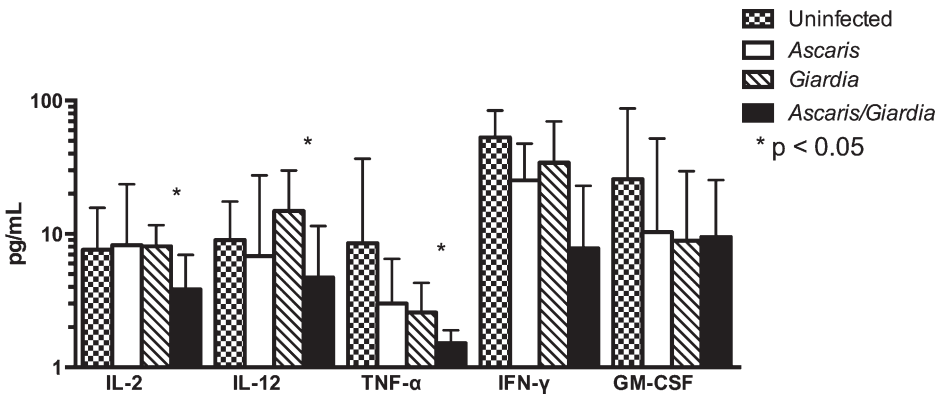


FIGURE 1. Th1-mediated cytokine repertoire in *Ascaris* and *Giardia* coinfection, *Ascaris*-only infection, *Giardia*-only infection, and uninfected. Decrease in IL-2, IL-12, and TNF-α in *Ascaris* and *Giardia* coinfected children compared with *Giardia*-only infected children. IL = interleukin; Th1 = T-helper cell 1; TNF-α = tumor necrosis factor alpha.

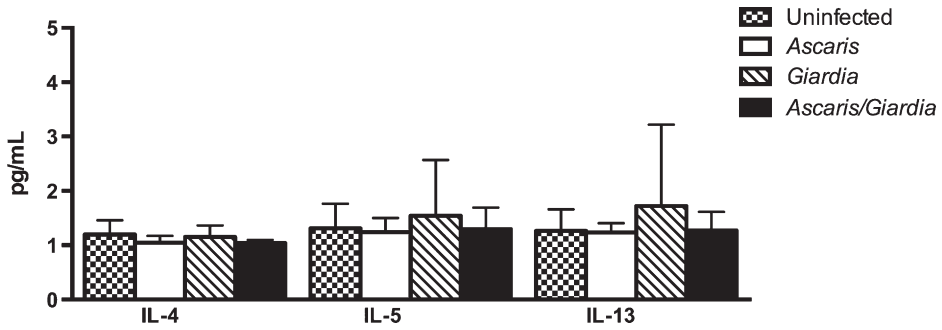


FIGURE 2. Th2-mediated cytokine repertoire in *Ascaris* and *Giardia* coinfection, *Ascaris*-only infection, *Giardia*-only infection, and uninfected. No difference was detected in *Ascaris* and *Giardia* coinfected children compared with *Giardia*- or *Ascaris*-only infected children. Th2 = T-helper cell 2.

of the immunomodulatory effects of ascariasis may impede the eradication of protozoa and permit persistent infections with *Giardia* in coinfected children.

IL-10, a product of innate immune cells, Tregs and B cells, is a potent anti-inflammatory cytokine.[17] IL-10 has been shown to inhibit IFN-γ-induced killing potential of macrophages against both intra- and extracellular parasites as well as prevent immune-mediated host tissue destruction in response to infectious agents.[17] The present study observed that children coinfected with *Ascaris* and *Giardia* had an increased IL-10/IFN-γ ratio (Th2/Th1 cytokines) compared with *Ascaris*-only infected children suggesting Th2 polarization. In a recent study of 1,060 children aged 4–11 years living in Brazil, those chronically infected with *A. lumbricoides* also demonstrated Th2 predominance with increased levels of Th2 cytokines including IL-5 and IL-10.[26] In a study by Sanchez and others, children coinfected with STHs in Honduras also had increased IL-10 concentrations compared with negative controls.[8] Previous studies have suggested that Th2 polarization and immunomodulation may be secondary to worm burden such that children with heavier helminth burden also have the highest levels of IL-10 concentrations in serum.[7,17] Furthermore, studies have shown that heavy *Ascaris* worm burden is associated with increased prevalence of *Giardia* coinfections.[7] However, despite demonstrating Th2 cytokine polarization in the current study, there was no significant difference in *Ascaris* burden between the *Ascaris* and *Giardia* coinfected group and the *Ascaris*-only group. There was also no statistically significant difference in concentrations of Th2 cytokines and IL-10 in plasma from children with *Ascaris* and *Giardia* coinfections compared with children infected with *Ascaris* only or *Giardia* only. Interestingly, there was no significant difference in hsCRP between the groups, and in fact hsCRP levels were comparable to those observed in U.S. children aged 3–16 years from the National Health and Nutrition Exam Survey study (mean value = 1.22 mg/mL).[27]

The complex interaction between parasitic coinfection and the immune system remains inconclusive. In a recent study by Blackwell and others, helminth infections and giardiasis were shown to have a negative association. *Giardia* infection was associated with lower odds of infection with *A. lumbricoides* (odds ratio [OR] = 0.63), and *A. lumbricoides* infection was associated with lower odds of *Giardia* (OR = 0.65). Interestingly, Blackwell and others did show that recovery from helminth infection after appropriate antihelminthic therapy was much less likely if the patient was coinfected with *Giardia* suggesting that the immunomodulation is occurring.[2] Recent studies have also shown the importance not only of $CD4^+$ producing Th1 cytokines, IFN-γ, and TNF-α, but also of $CD4^+$ producing IL-17A cells (Th17) in acute giardiasis. IL-17A is known to contribute to innate cell recruitment at the mucosal surface and, as a result, elevated levels of IL-17A during acute giardiasis may provide protection against development of chronic giardiasis.[18] Future studies evaluating the role of Th17 cells and IL-17A may provide additional knowledge on parasitic coinfections.

This study was limited by a sample size of 39 children and by high cost of the Luminex multiplex assays. Such a sample size would only have power to detect relatively

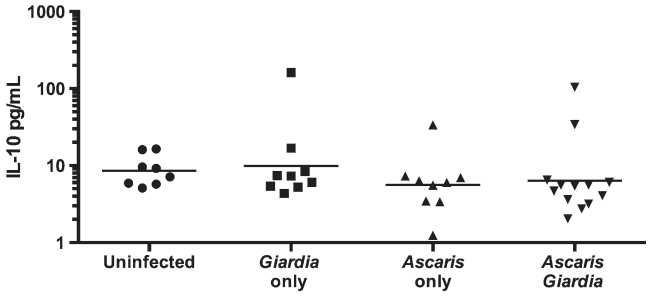


FIGURE 3. Th2-mediated cytokine repertoire in *Ascaris* and *Giardia* coinfection, *Ascaris*-only infection, *Giardia*-only infection, and uninfected children. No difference was detected in *Ascaris* and *Giardia* coinfected children compared with *Giardia*- or *Ascaris*-only infected children.

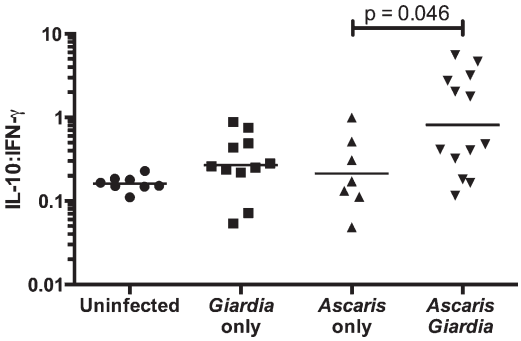


FIGURE 4. IL-10/IFN-γ ratio depicting increased Th2–Th1 cytokine polarization in *Ascaris* and *Giardia* coinfected children and *Ascaris*-only infected children. IL = interleukin; Th = T-helper cell; IFN = interferon.

large differences between infection groups, more likely to be biologically meaningful. Further analyses of the ECUAVIDA birth cohort will provide the opportunity to follow the children longitudinally to evaluate changes in cytokine production in acute versus chronic infection, cytokine production after treatment, and cytokine production upon reinfection. Figueiredo and others demonstrated that the production of IL-10 was greater in chronically infected children with *A. lumbricoides* than noninfected children or those in whom infection was detected at one observation time only.[26] Additionally, the study does not take into account the possibility of other infectious agents causing coinfection, such as viruses and bacteria, which may have significant Th1 and Th2 immunomodulatory effects. Both the *Ascaris* monoparasitic infected and the uninfected children did have elevated Th1 cytokine concentrations compared with the coinfected children suggesting that these children had additional inflammatory influence on the Th1 versus Th2 balance. However, these children were asymptomatic, with no reported diarrhea or abdominal symptoms at the time of stool collection. They did not have significantly elevated levels of the systemic inflammatory marker hsCRP compared with the uninfected group, also indicative of lack of clinically relevant concurrent bacterial or viral infections. There were no significant differences in baseline characteristics between the groups making confounding a less likely alternative explanation for our findings. These characteristics were chosen as factors known to be associated with the risk of infection at 3 years of age in a previous analysis of STH infections in the cohort.[28] However, as the effects of these potential confounders were not controlled or included in the analysis because of limited power, confounding cannot be excluded. Unfortunately, the measurement of plasma cytokines does not provide information regarding the specific cytokine-producing cells nor the tissue source. Previous studies of peripheral blood mononuclear cells have shown that STHs and intestinal protozoa may induce other types of host immune response such as Treg or Th17 cells during infection which have direct effects on both Th1 and Th2 responses.[13,29] Further investigation of the influence of antigen-specific Th17 cells, including IL-17A production, Tregs, including TGF-β, and additional Th2 cytokines such as IL-6 will contribute more depth to the peripheral cytokine profile landscape during *Ascaris* and *Giardia* coinfection in children.

## CONCLUSIONS

Parasitic coinfections in children remain a significant health problem around the world. STHs and intestinal protozoa have complex interactions with the host immune system that allow for persistent infection and subsequent chronic morbidity. In a similar study conducted in Venezuela, children with moderate *A. lumbricoides* infection had diminished antibody response and diminished pro-inflammatory cytokine production (Th1) when coinfected with *Giardia duodenalis* indicating ascariasis as a strong immunomodulating agent.[7] Additional studies evaluating the role of cytokines within the ECUAVIDA birth cohort suggest that chronic infection with STHs leads to cytokine hyporesponsiveness and a tolerized Th2 response further supporting the immunomodulatory role of STHs such as ascariasis.[30] Gaining a better understanding of this complex interaction between parasitic coinfection and the host immune system is essential to improve the long-term health outcome of children living in endemic areas through identification of therapeutic targets including vaccine development, alterations in environmental strategies, and shifts in public health policy.

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