Responses to Malarial Antigens Are Altered in Helminth-Infected Children

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Malaria and helminth infections often coincide in the same tropical regions. Studies of the consequences of helminth and malaria coinfection in humans have been few and are mainly epidemiological, with little information on cellular immune responses. In this study, we investigated the antimalarial immune responses of Ghanaian children living in a rural area with a high prevalence of both helminth infection and Plasmodium falciparum infection. Whole blood specimens were cultured with P. falciparum–infected red blood cells (iRBCs), and pro- and anti-inflammatory cytokines and immune regulatory molecules were measured. In response to iRBCs, levels of interleukin (IL)–10, but not tumor necrosis factor–α, were higher in samples from helminth-infected children than in those from uninfected children, as was expression of the regulatory molecules suppressor of cytokine signaling (SOCS)–3, Foxp3, and programmed death (PD)–1. Furthermore, a significant correlation was found between SOCS-3 gene expression and IL-10 production. These results indicate that the presence of helminth infection modulates the immune response to malarial parasites, making it more anti-inflammatory.

It is estimated that >1 billion persons worldwide are infected with parasitic helminths. Helminth infections are associated not only with Th2-skewed immune responses, but also with a strong regulatory network that is characterized by T cell hyporesponsiveness and production of anti-inflammatory cytokines, such as interleukin (IL)–10 [1]. The regulatory network seems to benefit both parasite and host, on the one hand by preventing the expulsion of the parasitic worms, prolonging their survival within their host [2], and on the other hand by protecting the host against the pathological consequences of inflammation [3, 4]. The immune hyporesponsiveness induced during chronic helminth infection affects responses not only to helminth antigens but also to bystander antigens. For example, chronic Onchocerca infection has been shown to lead to lower responses to tetanus [5] or to bacille Calmette-Guérin vaccination [6]. Furthermore, several studies have shown that helminth infections, such as schistosomiasis, are associated with reduced responsiveness to allergens [7].

Helminth infections are prevalent throughout the tropical regions where malaria parasites are transmitted. Coinfections with helminths and malaria parasites are frequently observed, and it is therefore important to consider that hyporesponsiveness caused by helminth infection might affect the immune response against malaria parasites and the course of infection. In murine models of malaria infection, concomitant infection with Schistosoma mansoni resulted in increased malaria parasite load [8]. Mice harboring a preexisting infection with the filarial worm Litomosoides sigmodontis show more severe malarial immunopathology after infection with Plasmodium chabaudi, accompanied by increased interferon (IFN)–γ responsiveness of spleen cells to polyclonal stimulation [9]. In contrast, inoculation with filarial larvae (L3) of Brugia pahangi protected mice against the development of Plasmodium berghei–induced cerebral malaria in another study [10]. Coinfection with the trematode Echinostoma caproni increased the fatality of Plasmodium yoelii infection in mice; IFN–γ

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production by polyclonally stimulated spleen cells was also reduced in coinfected mice, compared with mice infected with P. yoelii alone [11]. In humans, conflicting data have also been reported, with malaria-helminth coinfection either exacerbating [12, 13] or ameliorating the severity of malarial disease or parasite load [14, 15], indicating that additional studies are needed to resolve this issue.

There have been very few studies of immunological parameters during coinfection with helminths and Plasmodium falciparum. Some studies have looked at systemic serum cytokine levels in helminth-infected groups with and without P. falciparum [16, 17], but data are lacking on P. falciparum-specific cellular immune responses. In the present study, we examined immune responses against malarial antigens among children in a rural area of Ghana where malaria and helminth infections are highly prevalent, and we compared the groups with respect to their infection status, to clarify how helminth infections may alter responses to malarial antigens.

**SUBJECTS AND METHODS**

**Study population.** The study was conducted in rural schools in the Dangme East district in the Greater Accra Region of Ghana, where both helminth infection and malaria infection are endemic. Data from previous surveys in the area had indicated that the prevalence of helminth infections was ~55% and that of malaria infections was 50%. The Institutional Review Board of the Noguchi Memorial Institute for Medical Research, Accra, approved the study.

After study aims and procedures were explained, and after informed consent (signed by parents or guardians) was obtained, helminth infection was assessed in children aged 6–12 years. Blood specimens were obtained from helminth-positive and helminth-negative children within 1 week after parasitological assessment. The overall prevalence for helminth infection in the study population was 38%, resulting from infection with Ascaris species (14%), hookworm (11%), Schistosoma haematobium (11%), and Trichuris species (5%). The prevalence of P. falciparum infection was 46%, and all infected children had low-to-moderate parasite loads (i.e., <100 parasites/100 high-power fields [18]). None of the children had any symptoms of malaria, such as fever.

**Detection of helminth and malarial infection.** The participants were given specimen containers and were asked to collect fresh stool and urine samples for the detection of helminth infection. Stool specimen examination was performed by the Kato-Katz method for the detection of hookworm, Ascaris species, and Trichuris species, and the total number of eggs was calculated per gram of feces. Urine samples were tested for S. haematobium by passing 10 mL of urine through a filter with 10-μm pores. A subject was considered positive for helminth infection if eggs were detected for at least 1 helminth (hookworm, Ascaris species, or Trichuris species in stool or S. haematobium in urine). Blood samples were collected from all participants for the detection of malaria infection by Giemsa-stained thick smear examination.

**Malarial parasite antigen preparation.** P. falciparum asexual blood stages of the NF54 strain were cultured in vitro, as described elsewhere [19]. In brief, parasites were grown in Roswell Park Memorial Institute (RPMI) 1640 containing 10% human AB serum and a 5% hematocrit erythrocyte suspension in an automated continuous culture system. Parasites were cultured in the absence of antibiotics and were regularly screened for mycoplasmal contamination.

Asynchronous cultures of parasites were harvested at a parasitemia of approximately 10%–20%, and the mature asexual stages were purified by centrifugation on a 63% Percoll density gradient [20]. This purification step resulted in red blood cell (RBC) preparations with a 60%–80% parasitemia, consisting of >95% schizonts or mature trophozoites. These preparations of parasitized erythrocytes (infected RBCs [iRBCs]) were washed 3 times with phosphate-buffered saline (PBS), immediately frozen in cryotubes at −80°C and transported to Leiden on dry ice.

**Detection of cytokines.** Cytokine levels for IL-10, IL-6, TNF-α, and IFN-γ were measured by a Luminex assay using a Luminex 100 cytometer (Luminex) equipped with STarStation software, version 2.0 (Applied Cytometry Systems). Buffer reagent kits and Luminex cytokine kits (BioSource) were used, and cytokines were measured in accordance with standard protocol, with slight modifications [21], and were analyzed using the Luminex 100 cytometer. The lower detection limit of the assays was 3 pg/mL for IFN-γ, 5 pg/mL for IL-10 and IL-6, and 10 pg/mL for TNF-α. Samples with concentrations less than the
Messenger RNA (mRNA) expression analysis. In a subset of the samples (16 samples total), whole blood specimens obtained from children were stimulated not only for cytokine analysis but also for mRNA analysis in additional plates. Samples that showed a clear difference in production of IL-10 after stimulation with iRBCs were selected for RNA analysis (8 helminth-infected samples and 8 samples that were free of helminth infection). Supernatants were collected after 6, 16, and 24 h of whole-blood stimulation, and the remaining cells were mixed with 200 µL of ABI lysis buffer (Applied Biosystems) per well to stabilize the RNA and were stored at −80°C. The ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) was used for the isolation of RNA, using the whole-blood RNA-DNA protocol, which included a DNase step to remove contaminants of genomic DNA. Reverse transcription of RNA was performed using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen). Gene expression was assessed with real-time quantitative polymerase chain reaction (qPCR) in duplicate in accordance with the TaqMan assay instructions using TaqMan probes and qPCR Core kit reagents (both Eurogenetics) on an ABI 7500 machine (Applied Biosystems), normalizing for the housekeeping gene 18S rRNA [22].

Statistical analysis. Cytokine expression levels were not normally distributed, and differences between groups were tested with the nonparametric Mann-Whitney U test. Linear regression was performed with log-transformed values of the cytokines, because they were not normally distributed. Gene expression was compared between groups using the Mann-Whitney U test. Gene expression levels and cytokine production after stimulation with iRBCs were correlated by means of the nonparametric Spearman’s rank test, using absolute gene expression levels relative to the lowest value (not corrected for uRBC stimulation) and cytokine production without subtraction of values for uRBC stimulation. Differences were considered significant when P values were <.05.

RESULTS

Study population. In a pilot study in 2003, among a group of 20 urban and 20 rural children, we found that cells obtained from children living in a rural setting produced more IL-10 in response to malarial antigens than did cells from urban children [26]. Although the study population was rather small in the pilot study, the higher IL-10 production in response to P. falciparum appeared to be related to coinfection with helminths. Therefore, in the present study, more children living in a rural area where both malaria and helmhnt infections are endemic were recruited, to examine the effect that helminth infections may have on immune responses to P. falciparum (table 1). Helminth-infected children had S. haematobium and/or intestinal helminth infections and were slightly older than the uninfected children. Although the difference was not significantly different, helminth-infected children had a higher prevalence of P. falciparum infection than did those who were free of helmhnts (50% vs. 37%, respectively).

Production of IL-10 in response to malarial antigens. Whole-blood samples obtained from helminth-infected and -uninfected children were stimulated with P. falciparum iRBCs to investigate whether current helminth infection affected cytokine responses to malarial antigens. Both groups of children produced similar amounts of IL-6, TNF-α, and IFN-γ after stimulation with iRBCs, but little IL-5 production was seen in response to iRBCs. IL-10 response to malarial antigens was greater in helminth-
infected children \((P = .045)\), whereas IL-10 production in response to LPS did not differ significantly between infected and uninfected children (table 2 and figure 1A). There were no clear differences in IL-10 production after stimulation with iRBCs between children infected with \textit{S. haematobium} only and those infected with intestinal helminths only, nor was there a clear correlation between IL-10 production and the number of eggs (data not shown). In addition, multivariate linear regression analysis showed that age, sex, and \textit{P. falciparum} infection did not influence the association between IL-10 production and helminth infection.

### Expression of foxp3 and PD-1 in helminth-infected children after stimulation with malarial antigens.

It is known that chronic helminth infection can induce regulatory T cells that produce IL-10 \([27–29]\). The higher IL-10 responses to \textit{P. falciparum} we observed in helminth-infected individuals might be linked to the activity of T cells with a regulatory function. Therefore, in a subset of the samples, we measured the expression levels of Foxp3, a transcription factor associated with natural regulatory T cells, and PD-1, a member of the CD28

### Figure 1. Interleukin (IL–10) responses in whole blood stimulated for 24 h with lipopolysaccharide (LPS) or \textit{Plasmodium falciparum}–infected red blood cells (iRBCs). Samples were segregated according to helminth infection \((A)\) or malaria infection \((B)\). Open symbols represent uninfected subjects; closed symbols, infected subjects; horizontal lines, median values for group. The cytokine responses for each stimulus were compared between infected and uninfected samples, using the nonparametric Mann-Whitney \(U\) test.

#### Table 2. Cytokine responses to \textit{Plasmodium falciparum}–infected red blood cells (iRBCs) and lipopolysaccharide (LPS) in helminth-infected and helminth-free subjects.

<table>
<thead>
<tr>
<th>Cytokine, stimulation</th>
<th>Helminth-negative subjects ((n = 46))</th>
<th>Helminth-positive subjects ((n = 72))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion of responders ((%))</td>
<td>Median cytokine level in responders ((95% CI), \text{pg/mL})</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iRBCs</td>
<td>41/45 (91)</td>
<td>177 (101–694)</td>
</tr>
<tr>
<td>LPS</td>
<td>45/45 (100)</td>
<td>14100 (13,991–24,124)</td>
</tr>
<tr>
<td>IL-6: iRBCs</td>
<td>41/45 (91)</td>
<td>606 (701–1419)</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iRBCs</td>
<td>12/36 (33)</td>
<td>13 (6–21)</td>
</tr>
<tr>
<td>LPS</td>
<td>27/40 (68)</td>
<td>51 (12–90)</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iRBCs</td>
<td>40/45 (89)</td>
<td>13 (4–27)</td>
</tr>
<tr>
<td>LPS</td>
<td>45/45 (100)</td>
<td>640 (298–1544)</td>
</tr>
</tbody>
</table>

\(P\) values are given by the nonparametric Mann-Whitney \(U\) test.

\(\text{CI, confidence interval; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.} \)
family that negatively regulates T cell function and is expressed on regulatory T cells [30–32]. Twenty-four hours after stimulation with iRBCs, expression levels of both Foxp3 and PD-1 were significantly higher in the helminth-infected children than in -uninfected children (P = .02 and P = .01, respectively), and there was a similar trend with LPS stimulation (figure 2). Stimulation with LPS or iRBCs increased expression of SLAM, a gene included here as a control, but the levels did not differ between helminth-infected and -uninfected individuals (figure 2). This indicates that the up-regulation of Foxp3 and PD-1 in the helminth-infected group is not associated with an overall increase in gene expression of T cell markers. There were no differences in gene expression of PD-1, Foxp3, and SLAM between P. falciparum–infected and -uninfected individuals (data not shown).

**mRNA levels of SOCS-3 and SOCS-1 in helminth-infected children after stimulation with iRBCs.** SOCS-1 and SOCS-3 are known to influence the balance of cytokines produced after stimulation of immune cells [33]. Therefore, we measured the expression of SOCS-1 and SOCS-3 in cells obtained from helminth-infected and -uninfected children. After 16 and 24 h of stimulation with malarial antigens, SOCS-3 expression was significantly higher in cells obtained from helminth-infected children than in those from -uninfected children (P = .02 and P = .04, respectively), whereas no significant difference was observed after stimulation with LPS (figure 3). No significant differences in the expression levels of SOCS-3 were detected between P. falciparum–infected and -uninfected individuals (data not shown). Although the expression levels of SOCS-1 increased after stimulation with iRBCs or LPS, there were no significant differences between the levels of SOCS-1 expression in cells obtained from helminth-infected and -uninfected individuals at any time point (figure 3). Interestingly, there was a significant correlation between the gene expression levels of SOCS-3 and the levels of IL-10, but not of TNF-α, produced after stimulation with iRBCs (r = 0.60; P = .01) (figure 4).

**DISCUSSION**

In a rural area of Ghana, the cellular immune responses to P. falciparum antigens in helminth-infected children differed from those in children free of helminth infection. Helminth infection was associated with increased levels of IL-10 but not of the inflammatory cytokines IL-6 and TNF-α in response to malarial antigens. This modulation toward a more anti-inflammatory response was accompanied by higher mRNA expression of the known regulatory genes Foxp3, PD-1, and SOCS-3.

The development of pathology during malaria attacks is associated with an imbalance of cytokines involved in the regulation of inflammatory responses [34, 35]. Although proinflammatory...
responses are associated with protective immunity to malaria during the early phases of infection, overproduction of IFN-γ or TNF-α predisposes to severe malarial pathology [34, 36]. It has been reported that the anti-inflammatory cytokines IL-10 and TGF-β could have a protective effect against pathology [37–40]. The results of the present study suggest that helminth infections may modulate the antimalarial immune response through suppression of proinflammatory activity. Thus, the difference between the antimalarial responses in residents of urban and rural areas that we reported earlier [41] may reflect the greater frequency of helminths in rural areas.

It is also possible that, in rural areas, children’s intense, long-term exposure to plasmodial parasites can enhance regulatory mechanisms, because *P. falciparum* has been shown to induce

Figure 3. Suppressor of cytokine signaling (SOCS)–3 (A, C) and SOCS-1 (B, D) mRNA levels in whole-blood samples stimulated with *Plasmodium falciparum*–infected red blood cells (iRBCs) or lipopolysaccharide (LPS) for 6, 16, and 24 h, relative (rel) to levels after stimulation with uninfected RBCs or medium (values set to 1). Horizontal lines indicate median values for group. Open symbols represent uninfected subjects, and closed symbols represent helminth-infected subjects. *P < .05.

Figure 4. Scatter plots of gene expression levels for suppressor of cytokine signaling (SOCS)–3 sixteen h after stimulation with *Plasmodium falciparum*–infected red blood cells (iRBCs) and interleukin (IL)–10 (A) or tumor necrosis factor (TNF)–α (B) cytokine levels after stimulation with iRBCs for 24 h. Absolute gene expression levels after stimulation with iRBCs were used, relative (rel) to the lowest expression level, set to 1. For IL-10 levels, levels produced after stimulation with uninfected RBCs were not subtracted. Spearman’s rank test was used to test for correlation (*r* = 0.60, *P* < .05; NS, not significant). TNF, tumor necrosis factor.
regulatory T cells [42]. In the study presented here, however, the levels of IL-10, IL-6, and TNF-α produced in response to iRBCs by cells of children in rural areas did not differ between those with and those without *P. falciparum* infection. Nevertheless, IFN-γ responses were lower in *P. falciparum*-infected children than in -uninfected children, consistent with a recent report from Kenya. In the Kenyan study, malaria parasitemia in 1–6-year-old children was also associated with lower IFN-γ responses to the parasite antigen thrombospondin-related adhesion protein [43], but only prospectively and not with concurrent parasitemia, as in our study.

The mechanisms by which helminth infection may modify the immune response to the unrelated plasmodial parasite are not completely understood. Data from animal models have shown that helminth infections can lead to the induction of regulatory T cells that have the ability to inhibit both Th1 and Th2 responses directed to other pathogens [28, 29]. We have also shown that the schistosome lipid lysophosphatidylserine can modulate dendritic cells in vitro, driving polarization of naïve T cells to produce IL-10 and have suppressive activity [44]. High levels of regulatory cytokines that are induced by helminth and/or *P. falciparum* infection can create an environment that favors the induction of parasite-specific regulatory T cells. The higher levels of IL-10 production in response to iRBCs in the helminth-infected children indicate that regulatory T cells may be involved. Indeed, molecular analysis of gene expression showed that levels of Foxp3 and PD-1 expression were both higher in the helminth-infected group, indicating that T cells with a regulatory function might be preferentially induced in these children. Similarly, a recent study demonstrated that mice carrying the intestinal helminth *Heligmosomoides polygyrus*, compared with uninfected mice, had more cells with a regulatory phenotype, CD4+ CD25+ cells producing IL-10, in response to in vitro stimulation of spleen cells with malarial antigens [45]. More research at the cellular level is needed to determine the cellular source of the increased IL-10 levels.

The expression of SOCS-3, but not of SOCS-1, was higher in helminth-infected children than in -uninfected children after stimulation with malarial antigens. Modulation of SOCS-3 expression by exposure to malaria parasites might alter cytokine expression by antigen-presenting cells, such as monocytes and dendritic cells. Interestingly, the gene expression levels of SOCS-3 correlated with the amount of IL-10 produced after stimulation with malarial antigens. A number of recent studies have shown that IL-10 is, in fact, implicated in the induction of SOCS-3 [46, 47], which may be involved in mediating anti-inflammatory responses.

The majority of children we identified with *P. falciparum* infection were symptom free at the time of the study. It has been suggested that, although helminths might protect against severe malaria [48], the incidence of clinical malaria increases in helminth-infected patients [49]. Although we did find a tendency for a higher prevalence of *P. falciparum* parasitemia among helminth-infected children, different types of studies will be needed to assess the impact of helminth infections on malarial disease outcomes. Our results do indicate that chronic helminth infections change specific antimalarial immune responses to an anti-inflammatory profile, which could influence the development of immunopathology associated with inflammation. A fuller understanding of the impact of helminth and malaria coinfections on the immune system could be important in the prevention of severe malaria, the development of vaccination programs, and the evaluation of helminth eradication programs.

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