Short Report: Childhood Coinfections with *Plasmodium falciparum* and *Schistosoma mansoni* Result in Lower Percentages of Activated T Cells and T Regulatory Memory Cells than Schistosomiasis Only

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**Abstract.** Flow cytometric analyses were performed to evaluate HLA-DR+ activated T lymphocytes (Tact; CD3+/CD4+/CD25medium) and T regulatory cells (Treg; CD3+/CD4+/CD25high) in the circulation of children 8–10 years of age living in an area endemic for both *Plasmodium falciparum* and *Schistosoma mansoni* in western Kenya. Those children with only *S. mansoni* had a higher mean percentage of HLA-DR+ Tact than those who were co-infected with these two intravascular parasites. The proportion of circulating Treg was comparable in children with only schistosomiasis and both schistosomiasis and malaria. However, the mean level of memory Treg (Treg expressing CD45RO+) in those with dual infections was lower than in children with schistosomiasis alone. These imbalances in Tact and Treg memory subsets in children infected with both schistosomiasis and malaria may be related to the differential morbidity or course of infection attributed to coinfections with these parasites.

The parasites causing *Schistosomiasis mansoni* and falciparum malaria are co-endemic in parts of Africa.1,2 Also, the prevalence of these diseases is often highest in school children.3-9 Simultaneous infections with schistosome and *Plasmodium* species may impact the immunologic responses, the clinical manifestations, and possibly the development of acquired resistance to either parasite.4,10 Schistosomiasis is often acquired early in life (1–4 years of age) in Lake Victoria fishing villages,11 and if not treated, develops into a chronic infection.12,13 Malaria is acquired very early in life in holoendemic areas and, if a child survives beyond the fifth year, relative acquired immunity is established, although repeated sub-clinical and clinical infections can reoccur periodically. Although both of these infections are intravascular, schistosomiasis provides chronic antigen exposure, whereas malaria results in more episodic antigenic exposure. Because of the holoendemic nature of the area in which we did this study, the subjects may have had fairly frequent malarial episodes and thus somewhat more frequent exposure to malarial antigens. However, among 8- to 10-year-old children, natural worm death of their initial infections is not likely to have occurred frequently, if at all. This means their exposure to schistosome antigens will have been largely a result of chronic egg antigen exposure. Each of these parasites is known to stimulate both lymphocyte activation and T regulatory cell (Treg) involvement.14,15 In humans infected with *P. falciparum*, removal of Treg can enhance peripheral blood mononuclear cell proliferation and interferon (IFN)-γ responses to malaria antigen,16 which could indicate that Treg may play an important role in the control of immunopathology.

We studied immune profiles in children with schistosomiasis and malaria recruited from 8 primary schools in Bondo District, western Kenya, which is endemic for both *P. falciparum* and *S. mansoni*. The schools are all located within 3 km of the shores of Lake Victoria. A previous study indicated a prevalence of *S. mansoni* greater than 50% among 9- to 12-year-old children attending these schools.17 Data were collected between June and December 2006.

The Scientific Steering Committee of the Kenya Medical Research Institute, the National Ethics Review Board of Kenya, and the Institutional Review Boards of the University of Georgia and the Centers for Diseases Control and Prevention approved the investigation. After obtaining written informed consent from parents and assent from children, 2 slides from 1 stool specimen were examined for *S. mansoni* eggs and other helminth ova by the modified Kato-Katz method (Vestergaard-Frandsen, Denmark). Data were recorded as eggs per gram (EPG) of stool. Thick and thin blood smears of finger prick blood were examined for malaria parasites. Those few children with malaria caused by *Plasmodium* spp. other than *P. falciparum* were not included in this study. Participants positive for *S. mansoni* were further consented to take part in immunologic studies. When diagnosed with any of the soil-transmitted helminths or schistosomiasis, the children were offered treatment with albendazole (400 mg) and/or praziquantel (PZQ; 40 mg/kg; Cosmos, Ltd., Nairobi, Kenya), respectively. Participants who were blood smear-positive for malaria were offered appropriate therapy with CoArtem (Beijing Novartis Pharma, Ltd., Beijing, China). None of the children had been previously treated with PZQ. These current findings are initial baseline observations from an ongoing longitudinal protocol to evaluate the immunologic impact of multiple treatments for schistosome reinfections acquired during childhood.

Blood for flow cytometric analyses was collected by venipuncture into EDTA-coated glass tubes (BD Vacutainer System, Franklin Lakes, NJ). To identify Treg in peripheral blood, anti-CD3, anti-CD4, and anti-CD25 (e-Bioscience, San Diego, CA) were used. In our previous study of Treg in adults with schistosomiasis,15 we defined Treg as the CD3+/CD4+/CD25high population.18-24 These longitudinal studies were started before the commercial availability of appropriate FoxP3 reagents that identify CD4+/CD25high functional Treg.25 Activated T cells (Tact) were defined as the CD3+/CD4+/CD25medium cells and those that also expressed HLA-DR+ (e-Bioscience,
San Diego, CA) were considered highly activated.\textsuperscript{13} We first gated to separate CD25\textsuperscript{positive} cells from CD25\textsuperscript{negative} cells based on fluorescence minus one (FMO) stained samples.\textsuperscript{26,27} To distinguish CD25\textsuperscript{hi} from CD25\textsuperscript{lo} cells, we examined the CD4/CD25 dot plots from all patients studied and set thresholds (Figure 1) that most often differentiated these subpopulations (CD25\textsuperscript{lo}, CD25\textsuperscript{med}, CD25\textsuperscript{hi}). These thresholds were then used for all data analyses. To examine phenotypic subsets of Treg, anti-PD-1 (CD279), and anti-CD45RO (BD Pharmingen, San Diego, CA) were used and gating performed using FMO-stained samples. For statistical analyses the Kruskal-Wallis non-parametric post-test was used following analysis of variance (ANOVA) analysis when 3 or more groups were compared. The Mann-Whitney non-parametric \textit{t} test was used when 2 groups were compared. Comparisons with \textit{P} values ≤ 0.05 were considered statistically significant.

A total of 153 school children (8–10 years of age) with \textit{S. mansoni} infection were included in the study. Group sample sizes differ for different tests because of the unavailability of some samples from some children. Children were stratified into three levels of infection intensity based on \textit{S. mansoni}: low (< 100; \textit{N} = 89), medium (100–399; \textit{N} = 41), and high (≥ 400; \textit{N} = 23). The demographic, clinical, and laboratory characteristics of the study participants are presented in Table 1. Malaria prevalence was comparable in the low and medium intensity \textit{S. mansoni} groups (29%, and 30%, respectively) and lower in the high intensity group (10.5%), although this difference was not significant compared with the other 2 groups. There were no significant differences in age, hemoglobin concentrations, height, weight, or prevalence of other helminth infections (Ascaris, hookworm, and \textit{Trichuris}) between the groups (Table 1).

We found no difference in the percentage of circulating CD3/CD4\textsuperscript{+} cells in children with schistosomiasis only when compared with those in children with both schistosomiasis and malaria (55.4 ± 1.0 versus 55.4 ± 1.7; mean ± SEM, respectively).

We examined the level of circulating Tact cells in the children with only \textit{S. mansoni} infections (\textit{N} = 90) and found that the mean percentage of these cells that also expressed HLA-DR\textsuperscript{+}, indicating highly activated T cells,\textsuperscript{28} was 16.1%. However, when this Tact (HLA-DR\textsuperscript{+}) subset was analyzed in the circulation of children with \textit{S. mansoni} and \textit{P. falciparum} coinfections (\textit{N} = 32), the level was significantly lower (5.2%; \textit{P} = 0.0173; Figure 2A). This significantly lower level of circulating HLA-DR\textsuperscript{+}, highly activated T lymphocytes in children coinfected with schistosomes and \textit{P. falciparum}, compared with schistosomes alone, may indicate that responses to coinfections led to either decreased T cell activation or immune sequestration of HLA-DR\textsuperscript{+} Tact from the circulating lymphocyte pool.

The percentages of Treg did not differ statistically when compared on the basis of children’s ages or when children with schistosomiasis alone (\textit{N} = 122) were compared with those co-infected with both \textit{S. mansoni} and \textit{P. falciparum} (\textit{N} = 32) (data not shown). The mean percentage of Treg in the circulation of children with schistosomiasis only was 2.1% (SD ± 1.3). We have previously reported the mean Treg percentage in adults with chronic patent \textit{S. mansoni} infections is 4.7%, and approximately 31% of adult \textit{S. mansoni} patients have 5–10% circulating Treg and another 9% have very high Treg percentages (> 10%).\textsuperscript{15} The percent of circulating Treg in uninfected adults is usually 1–3%;\textsuperscript{24} Thus, 8- to 10-year-old children with \textit{S. mansoni} infection exhibit circulating Treg levels that are comparable to those of uninfected adults and appear lower than adults with long-standing patent \textit{S. mansoni}. The percentages of Treg in the peripheral blood were analyzed based on low, medium, or high numbers of EPG of feces, but as with infected adults,\textsuperscript{15} there were no significant differences in Treg percentages between the three groups (data not shown). Likewise, the intensity of a child’s schistosomiasis infection did not lead to significant differences in either the percentage of Treg that expressed the immunosuppressive marker PD-1, or mean fluorescence intensity PD-1 expression levels (data not shown).

There was, however, a significant difference (\textit{P} = 0.0030; Figure 2B) between the mean percentage of Treg that expressed the T cell memory marker CD45RO\textsuperscript{+} based on whether children had only schistosomiasis or coinfection with both \textit{S. mansoni} and falciparum malaria (Figure 2B).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1.png}
\caption{Flow cytometry analysis of whole blood cells gated on CD3+CD4+ cells within the lymphocyte gate, stained with anti-CD25 and used to define CD3+CD4+/CD25\textsuperscript{lo}, CD3+CD4+/CD25\textsuperscript{med}, and CD3+CD4+CD25\textsuperscript{hi} cells. Staining gates for CD3, CD4, and CD25 were determined using thresholds based on the fluorescence minus one (FMO) “no stain” controls. After examination of the scatter plots of all patients, a threshold was set between CD25\textsuperscript{med} and CD25\textsuperscript{hi} cells (line between the blocks denoting Tact and Treg, respectively). These thresholds, and those provided by the FMO controls, were then used throughout for all analyses.}
\end{figure}

\begin{table}[h]
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\begin{tabular}{lccc}
\hline
\textbf{Parameter} & \textbf{Low intensity*} & \textbf{Medium intensity*} & \textbf{High intensity*} \\
\hline
Number of participants & 89 & 41 & 23 \\
Gender (n, % Male) & 46 (51.7) & 21 (51.2) & 14 (60.9) \\
Age (year) (mean ± SD) & 9.2 ± 0.9 & 9.3 ± 0.9 & 9.6 ± 0.6 \\
Hemoglobin (g/dL) (mean ± SD) & 11.2 ± 1.6 & 11.6 ± 1.4 & 11.1 ± 2.1 \\
Plasmodium falciparum (%) & 29.0 & 30.3 & 10.5 \\
Other helminths (%) & 38.2 & 43.9 & 43.5 \\
Height (cm) (mean ± SD) & 130.3 ± 8.6 & 131.5 ± 8.6 & 133.7 ± 1.2 \\
Weight (kg) (mean ± SD) & 27.8 ± 5.2 & 28.8 ± 5.4 & 29.7 ± 4.2 \\
\hline
\end{tabular}
\caption{Demographic, clinical, and laboratory characteristics of the study participants categorized based on levels of \textit{Schistosoma mansoni} eggs per gram of feces.}
\end{table}

\textsuperscript{*} Low (1–99 eggs per gram [EPG]), Medium (100–399 EPG), and High (≥ 400 EPG).
mean percentage of CD45RO⁻ Treg (memory Treg) in children with S. mansoni infection alone was 71.2%, whereas it was 60.3% in those with both infections. This lower level of circulating Treg memory cells suggests that coinfection of schistosome-infected children with falciparum malaria may lead to decreased immunoregulatory capacity, or differential margination of memory Treg.

Analyses of T lymphocyte subsets based on patent infections with any of the three soil-transmitted helminths failed to yield statistically significant differences in terms of coinfections with schistosomiasis (data not shown).

Children in rural western Kenya and many other parts of sub-Saharan Africa are at risk of simultaneously harboring multiple infectious diseases. The goal of this baseline study was to determine whether coinfection of primary school children with S. mansoni and P. falciparum alters the levels of certain T lymphocyte subsets, as compared with children with S. mansoni alone. We have found that children with coinfections of S. mansoni and falciparum malaria present with alterations in their circulating highly Tact and Treg memory subsets. Others have reported on children with coinfections with both parasites, and have found differences in morbidity, and possibly resistance to reinfections with either of the parasites compared with infection with only one parasite. In fact, Wilson, and others have shown that these coinfections lead to decreased Th2 cytokine responses to egg antigens, whereas the reverse was true for TNF-α production. Furthermore, these coinfection-mediated, immunologic alterations correlated with the degree of hepatomegaly. It is plausible that a stabilized heightened immune system resulting from chronic antigenic exposure from patent schistosomiasis might be influenced by the episodic immune disturbances characterized by bouts of clinical or sub-clinical falciparum malaria. These studies begin to evaluate those immune parameters often associated with overactivation or immunoregulation because of these infections, and we anticipate our ongoing, longitudinal studies of these and other immune markers will allow increased understanding of both protective and morbidity-related immune responses caused by both schistosomiasis and malaria in children living in co-endemic areas.

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