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Praziquantel treatment shows that down-regulation of antigen specific immune responses in human schistosomiasis is associated with $CD4^+CD25^+FOXP3^+$ T cells.

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Author Summary

Schistosomiasis is a chronic infection that affects over 200 million people in particular children in developing countries. To answer the question how infection affects the immune system, we conducted a longitudinal study in schoolchildren with and without *Schistosoma haematobium* infection before and after treatment with praziquantel living in an endemic area in Gabon. We used whole blood assays to investigate innate immune responses, by stimulating pattern recognition receptors, and adaptive immune responses, by stimulating with schistosomal antigens. We also characterized the $CD4^+$ T cell memory compartment and assessed the frequency of $CD4^+CD25^+FOXP3^+$ T cells. After removal of *S. haematobium* infection by praziquantel, the most prominent effect was seen on parasite antigen-specific cytokine responses. An increase was seen in schistosome-specific antigen responses following treatment, which was inversely associated with a decrease in $CD4^+CD25^+FOXP3^+$ T cells. These results for the first time provide evidence for the association between schistosome infection, regulatory T cells and antigen specific immune hypo-responsiveness in humans.

Abstract

Background: *Schistosoma haematobium* infection results in alterations in immune function, yet the concurrent interplay between the various arms of the immune system and the effect of parasite removal following anthelmintic treatment has not been studied extensively. We conducted a longitudinal study to investigate the effect of *S. haematobium* infection and its clearance on innate and adaptive immune responses, on $CD4^+$ T cell memory compartment and on $CD4^+CD25^+FOXP3^+$ T cells.

Methods: Cytokine responses of Gabonese schoolchildren infected with *S. haematobium* (n=40) were studied in whole blood at pre- and 7 months post-praziquantel (PZQ) treatment; responses of uninfected children (n=39) were studied at the two time points as controls. To assess adaptive immune responses, the production of interferon gamma (IFN-γ), interleukin-2 (IL-2), IL-5, IL-10 and tumor necrosis factor (TNF) was measured after stimulation with schistosome soluble egg and adult worm antigens and the mitogen PHA. Innate immune responses were assessed by the production of IL-10, IL-1β, IL-1ra and TNF following culture with Pam3, mannan and curdlan, alone or in combination. Cytokines in supernatants were quantified by Luminex. Flow cytometry was used to characterize the memory T cell compartment and to assess $CD4^+CD25^+FOXP3^+$ T cell frequencies.

Results: Schistosome-specific responses increased in *S. haematobium*-infected schoolchildren following treatment with PZQ, but the effect of removal of infection on innate immune responses was marginal. Furthermore PZQ treatment resulted in increased effector memory T cells (T_{EM}) in infected schoolchildren. $CD4^+CD25^+FOXP3^+$ T cell frequencies, which were higher in infected children, decreased following anti-schistosome treatment. In *S. haematobium*-infected children there was a significant negative association between the decrease in $CD4^+CD25^+FOXP3^+$ T cell frequencies and the increase in schistosome-specific IL-5 and IL-10 cytokine levels observed upon treatment.

Conclusions: This study supports the notion that schistosome infections in humans can lead to the expansion of $CD4^+CD25^+FOXP3^+$ T cells leading to down-regulation of schistosome-specific immune responses.

Introduction

Schistosomiasis, caused by the parasitic trematodes of the genus *Schistosoma*, affects over 200 million people worldwide, especially children [1]. Chronic infections with schistosomes are associated with immune hypo-responsiveness characterized by reduced in vitro proliferation of T lymphocytes and decreased Th1- and Th2-type cytokine production in response to not only schistosomal antigens [2] but also to third party antigens such as Mycobacterium tuberculosis purified protein derivative (Mtb PPD) [3] and the influenza vaccine [4]. Treatment with antischistosomal drug praziquantel leads to clearance of infection [5] and elevated antigen-specific proliferation [6] and cytokine production [7,8]. The mechanisms of T cell hypo-responsiveness during chronic schistosomiasis are not well understood, but a number of recent reports have shown that in addition to impaired dendritic cell activity during schistosome infection [9], regulatory T cells [10] and regulatory B cells [11] are expanded in schistosome-infected compared to uninfected individuals. However, few studies have examined the effect that parasite elimination by chemotherapy has on $CD4^+CD25^+FOXP3^+$ T cells and how this is linked to effector T cell responses.

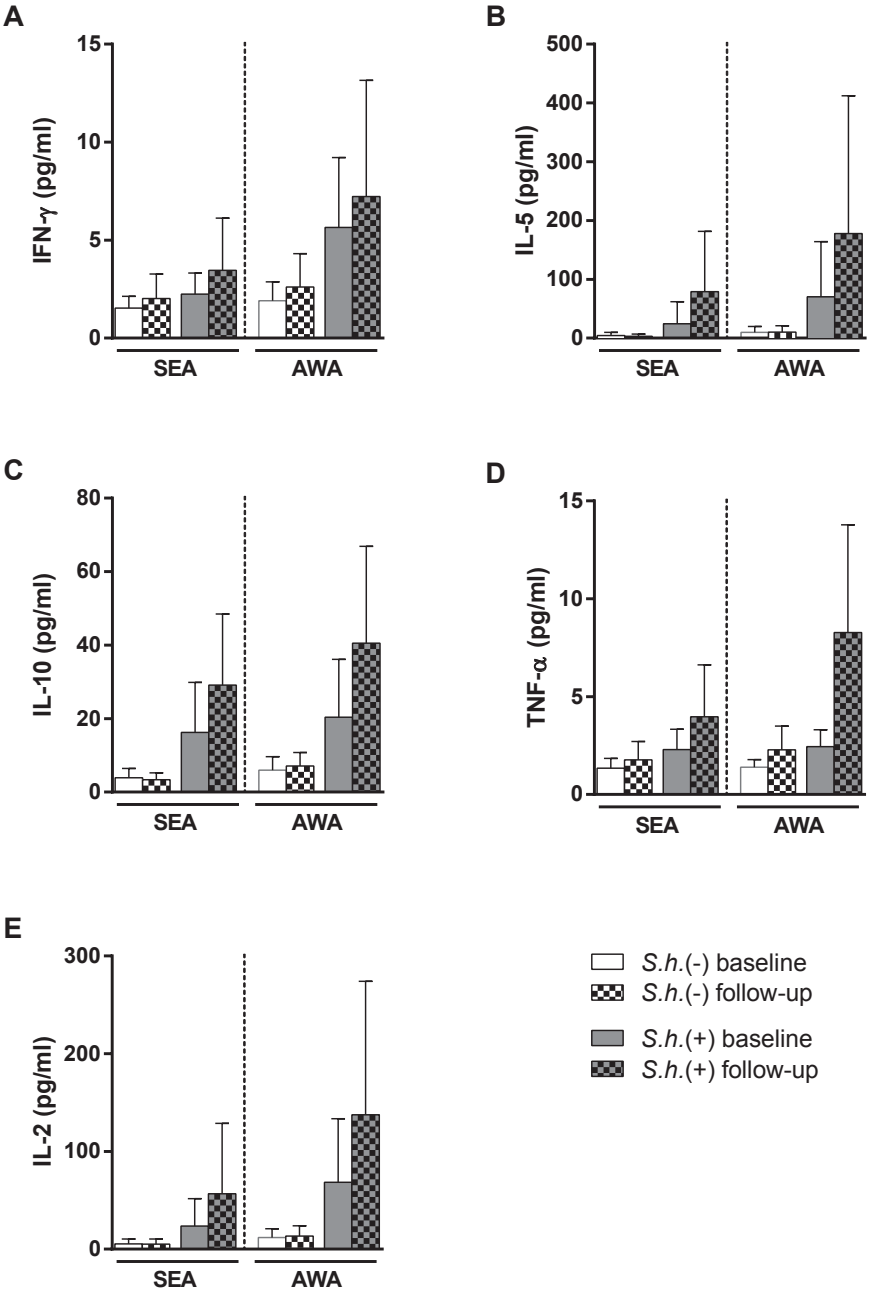
Moreover there are very few studies on innate immune responses in the context of human schistosomiasis. Responses to and expression levels of the Toll-like receptors (TLRs) have been shown to be altered in *Schistosoma haematobium*-infected individuals. For example, myeloid dendritic cells (mDCs) from infected individuals had reduced capacity to respond to TLR ligands [9], PBMCs from infected children produced higher levels of TNF in response to TLRs [12], but TLR2 expression was significantly reduced in children infected with schistosomes compared to uninfected age-matched individuals [13]. Another class of pattern recognition receptors (PRRs), the C-type lectins (CLRs) which includes DC-SIGN and mannose receptor (MR), has also been shown to be important in the recognition of schistosomal ligands [14]. It has been proposed that innate immune responses could be fine-tuned via interaction between distinct PRRs [15,16]. For example schistosomal glycolipids activate DCs to produce inflammatory cytokines through the cooperation of TLR4 and DC-SIGN [17]. However, altogether, little is known about innate immune responses in humans during the course of schistosome infection and the data available come from studies that were cross-sectional in design.

To our knowledge there are no reports that have examined, within one study, the effect of schistosome infection on different components of the immune system. Here we have conducted a longitudinal study where both adaptive and innate immune responses were examined in *S. haematobium*-infected individuals before and after treatment with praziquantel. To this end, we have analyzed cytokine responses to schistosomal antigens as well as to stimuli that trigger PRRs. In order to assess how schistosome infection affects the different components of the adaptive immune system, we have characterized the $CD4^+$ T cell memory compartment and have determined the frequency of $CD4^+CD25^+FOXP3^+$ T cells during infection and after removal of schistosomes by chemotherapy.

Table 1: Characteristics of the study population.

	S. haematobium uninfected	*S. haematobium* infected	p value
Participants (n)	39	40	
Mean age in years (range)	11.31 (6 - 15)	12.73 (9 - 20)	0.006[a]
Sex (male/female)	24/15	17/23	0.117[b]
P. falciparum infection (n/total)	19/39	18/40	0.823[b]
Intestinal helminth infection (n/total)	30/39	32/39	0.780[b]
A. lumbricoides infection (n/total)	17/39	15/39	0.818[b]
T. trichiura infection (n/total)	24/39	24/39	1.000[b]
Hookworm infection (n/total)	14/39	15/39	1.000[b]
Erythrocyte sedimentation rate (mm/h): mean (95% CI)	23.14 (6.70 - 57.25)	20.10 (2.00 - 51.00)	0.372[a]
Level of white blood cell ($x10^3$/µL): mean (95% CI)	8.39 (4.69 - 13.02)	9.12 (6.10 - 13.84)	0.182[a]
Level of lymphocytes ($x10^3$/µL): mean (95% CI)	3.20 (1.51 - 4.59)	3.42 (2.12 - 5.28)	0.271[a]
Level of monocytes ($x10^3$/µL): mean (95% CI)	0.84 (0.36 - 2.00)	0.76 (0.47 - 1.45)	0.332[a]
Level of neutrophils ($x10^3$/µL): mean (95% CI)	2.80 (1.07 - 4.74)	2.72 (1.74 - 3.82)	0.765[a]
Level of eosinophils ($x10^3$/µL): mean (95% CI)	1.09 (0.13 - 2.55)	1.37 (0.42 - 3.02)	0.174[a]
Level of basophils ($x10^3$/µL): mean (95% CI)	0.07 (0.03 - .15)	0.07 (0.03 - .14)	0.707[a]
Level of platelets ($x10^3$/µL): mean (95% CI)	255.17 (106.50 - 374.10)	245.92 (113.40 - 420.00)	0.623[a]
Level of haemoglobin (g/dL): mean (95% CI)	11.89 (10.50 - 13.34)	11.78 (10.35 - 13.68)	0.597[a]

CI, confidence interval. Infections are depicted as number of participants infected out of total number of participants tested. [a]Independent student's T-test; [b]Fisher's Exact Test.


A
IFN-γ (pg/ml)
15
10
5
0
SEA
AWA
B
IL-5 (pg/ml)
500
400
300
200
100
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SEA
AWA
C
IL-10 (pg/ml)
80
60
40
20
0
SEA
AWA
D
TNF-α (pg/ml)
15
10
5
0
SEA
AWA
E
IL-2 (pg/ml)
300
200
100
0
SEA
AWA
S.h.(-) baseline
S.h.(-) follow-up
S.h.(+) baseline
S.h.(+) follow-up

Results

Study population characteristics

We recruited *S. haematobium*-infected (N = 40) and -uninfected (N = 39) schoolchildren; Table 1 shows the demographic, parasitological and hematological characteristics of the two study groups. There were no significant differences between these two groups in the prevalence of *P. falciparum*, *A. lumbricoides*, *T. trichiura* and hookworm or in hematological parameters at baseline. Similarly, the number of females and males was comparable but infected children were approximately one year older. Schistosome infection parameters as assessed by egg counts, plasma CAA, hematuria and RSI score are given in Table 2; and as expected all were significantly correlated with each other ($p < 0.001$). Praziquantel treatment significantly reduced all infection parameters in the infected schoolchildren ($p < 0.001$; Table 2).

Cytokine responses to schistosomal antigens and mitogen

Age-adjusted mean cytokine levels following whole blood stimulation with SEA and AWA before and 7 months after praziquantel treatment are shown in Figure 1 and Table 3. At baseline, *S. haematobium* infected schoolchildren responded to SEA and AWA, by producing IFN-γ,IL-2, IL-5, IL-10 and TNF while uninfected controls did not (Figure 1). There were no differences in response to PHA between infected and uninfected controls. Following removal of worms by treatment, cytokine responses were enhanced. Specifically, IL-5, IL-10 and IL-2 levels in response to SEA and IL-5, IL-10 and TNF levels in response to AWA were significantly increased in *S. haematobium*-infected children (Figure 1 and Table 3), as was IL-10 in response to PHA ($p=0.001$). No differences were observed in the uninfected control children between baseline and 7 month follow-up time point. In addition, no differences were seen in spontaneous cytokine production (in unstimulated cultures) between infected and uninfected children or between pre- and post-treatment responses (Supplementary figure 1B).

Cytokine responses to TLR and CLR ligands

There were no differences in cytokine responses to Pam3 (TLR1/2 ligand) between *S. haematobium*-infected schoolchildren and uninfected controls at baseline (Figure 2). Over the 7 month study period, the levels of IL-1β and IL-10 decreased significantly in all study subjects, which might reflect the decrease that was seen in *P. falciparum* infection and/or infection load in both *S. haematobium* infected (baseline 18/40 infected vs. follow-up 10/38 infected; $p=0.011$) and uninfected controls (baseline PCR ct value 39.25 vs. follow-up 35.34; $p=0.024$)

Figure 1: Cytokine production in response to schistosomal antigens in whole blood cultures at baseline and 7 months follow-up.

Whole blood from *S. haematobium* infected schoolchildren and uninfected controls was stimulated with schistosomal egg antigen (SEA) and adult worm antigen (AWA). After 72 hours of culture levels of Th1 (A), Th2 (B), regulatory (C), pro-inflammatory (D) and IL-2 (E) cytokines were measured by Luminex. The follow-up samples were obtained at 7 months post-treatment with praziquantel of children that were infected with *S. haematobium* at pre-treatment. The uninfected controls were also examined at the same follow-up period but did not receive praziquantel.

Table 2: Median values of infection indices in the study population.

	Baseline		Follow-up	
	S. haematobium uninfected	*S. haematobium* infected	*S. haematobium* uninfected	*S. haematobium* infected
Median egg counts (IQR)	0.0 (0.0 – 0.0)[a]	19.5 (5.3 – 87.0)[a,b]	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)[b]
Plasma CAA levels pg/ml (IQR)	0.0 (0.0 – 3.0)[a]	420.5 (12.3 – 1196.3)[a, b]	0.0 (0.0 – 0.3)	12.5 (0.0 – 74.3)[b]
Haematuria score (IQR)	0.0 (0.0 – 0.0)[a]	3.0 (0.0 – 3.0)[a, b]	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)[b]
RSI score (IQR)	0.0 (0.0 – 2.0)[a]	5.0 2.0 – 6.3)[a, b]	0.0 (0.0 – 2.0)	0.0 (0.0 – 1.0)[b]

CAA, circulating anodic antigen; RSI, reagent strip index; IQR, interquartile range. Differences between groups were tested with Mann-Whitney U test and within group differences between baseline and follow-up with Wilcoxon matched pairs test. Significant between group differences are indicated with [a] and within group differences with [b]; all p<0.001. Infection indices were all significantly correlated with each other: egg counts with hematuria ($\rho = 0.767$, $p < 0.001$); egg counts with RSI ($\rho = 0.746$, $p < 0.001$); CAA with hematuria ($\rho = 0.632$, $p < 0.001$); CAA with RSI ($\rho = 0.556$, $p < 0.001$); and egg counts with CAA ($\rho = 0.748$, $p < 0.001$).

Table 3: Changes in cytokine production in response to schistosomal products in whole blood cultures 7 months after praziquantel treatment.

		S. haematobium uninfected		*S. haematobium* infected	
Cytokine	Stimulus	Post-/Pre-tx ratio (95% CI)	p value	Post-/Pre-tx ratio (95% CI)	p value
IFN-γ	SEA	1.25 (0.78 - 2.00)	0.332	1.67 (1.00 - 2.78)	0.051
	AWA	1.32 (0.82 - 2.14)	0.243	1.34 (0.71 - 2.51)	0.339
IL-5	SEA	0.69 (0.28 - 1.71)	0.408	3.60 (1.92 - 6.76)	0.001[a]
	AWA	1.03 (0.56 - 1.90)	0.922	2.67 (1.78 - 4.00)	<0.001[a]
IL-10	SEA	0.81 (0.45 - 1.47)	0.476	2.00 (1.34 - 2.98)	0.002[a]
	AWA	1.16 (0.63 - 2.11)	0.623	2.07 (1.41 - 3.03)	0.001[a]
TNF	SEA	1.25 (0.85 - 1.84)	0.235	1.84 (1.08 - 3.14)	0.027
	AWA	1.65 (1.16 - 2.37)	0.008	3.34 (1.65 - 6.76)	0.002[a]
IL-2	SEA	0.90 (0.46 - 1.76)	0.758	2.61 (1.68 - 4.07)	<0.001[a]
	AWA	1.11 (0.57 - 2.16)	0.744	2.03 (1.14 - 3.61)	0.020

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; SEA, schistosomal egg antigen; AWA, adult worm antigen; tx, treatment. Differences within groups were tested with a paired samples T test and the resulting mean differences were anti-log-transformed to obtain the before/after treatment ratio. [a]significant change after α-adjustment by the Bonferroni-Holm method.

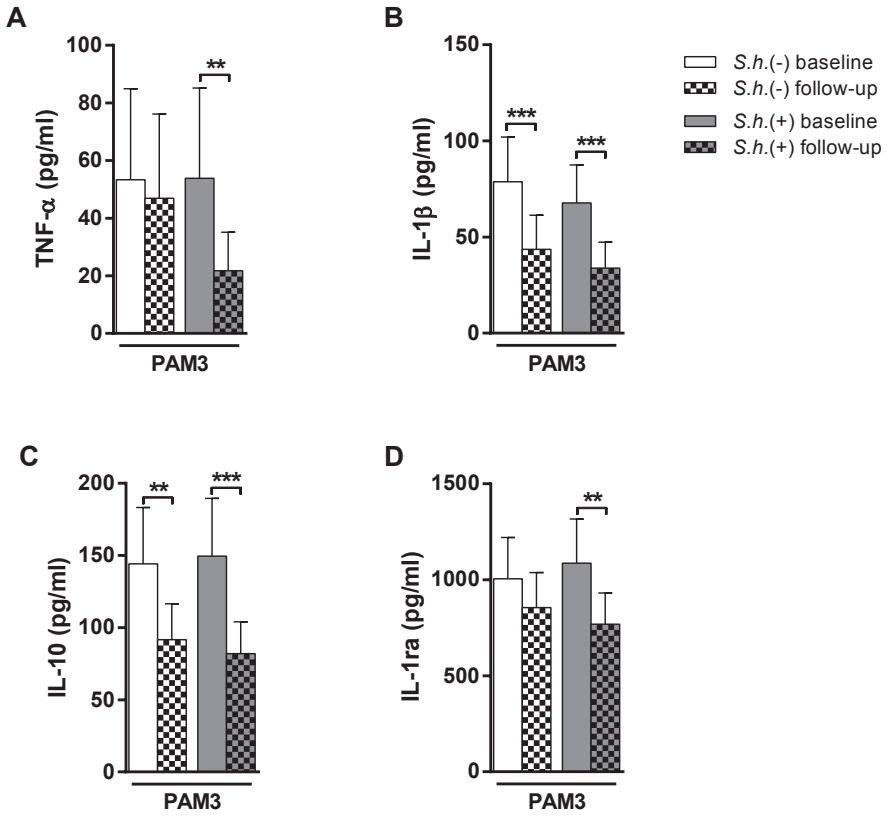

Figure 2: Cytokine production in response to TLR2/1 stimulation in whole blood cultures at baseline and 7 months follow-up.

Whole blood from *S. haematobium* infected schoolchildren and uninfected controls was stimulated with Pam3 (TLR2/1). After 24 hours of culture TNF (A), IL-1β (B), IL-10 (C) and IL-1RA (D) were measured by Luminex. Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as age-adjusted geometric means. * p< 0.05, ** p< 0.01, *** p< 0.001.

over time. However, TNF response to Pam3 decreased significantly, after treatment of *S. haematobium* infected subjects, a response that was different from that observed in uninfected controls, indicating that removal of *S. haematobium* infection is associated with decrease in TNF production in response to Pam3. Responses to mannan (DC-SIGN/MR ligand) and curdlan (Dectin-1 ligand) alone were largely not detectable (data not shown). However when combined with Pam3, significant synergy for pro-inflammatory responses (TNF and IL-1β) was observed. For anti-inflammatory/regulatory responses (IL-10 and IL-1ra) combined stimulation of Pam3 with mannan led to synergistic increase but with curdlan resulted in inhibition (Supplementary table 1). There were no significant differences between *S.haematobium*-infected and uninfected schoolchildren in these interactions. No differences in unstimulated responses were observed between infection groups or between pre- and post-treatment responses (Supplementary figure 1A).

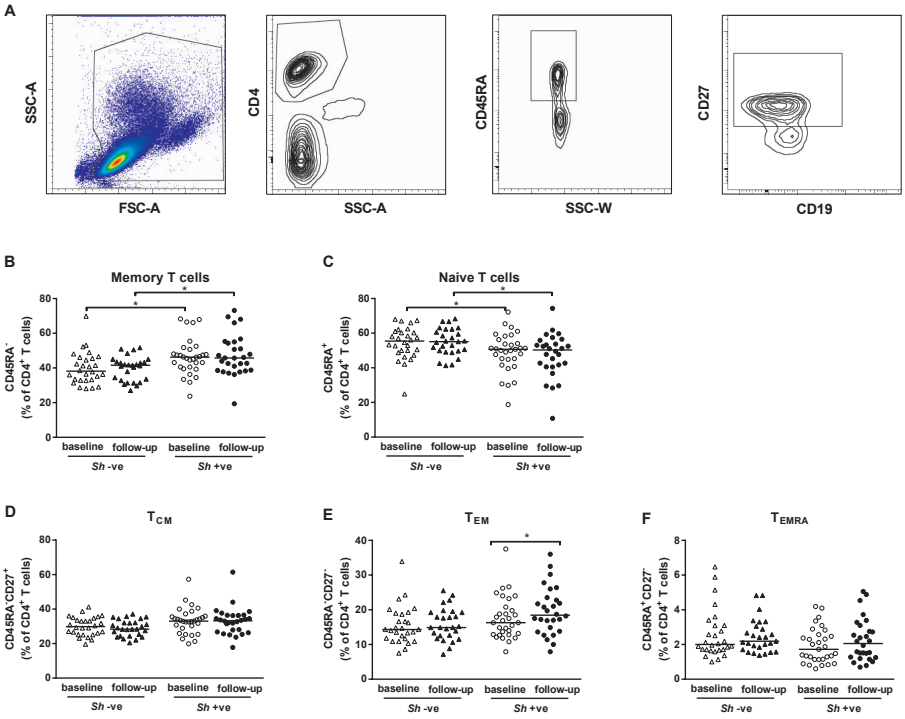

Figure 3: Memory T cell analysis at baseline and 7 months follow-up.
$CD4^+$ T cells were identified and Boolean gating combinations were used to determine proportions of T cell subsets based on CD45RA and CD27 expression (A). Proportion of $CD4^+$-gated cells that were $CD45RA^-$ (B, memory), $CD45RA^+$ (C, naïve), $CD45RA^-CD27^+$ (D, T_{CM} early), $CD45RA^-CD27^-$ (E, T_{EM}) and $CD45RA^+CD27^-$ (F, T_{EMRA}) were determined for *S. haematobium*-infected and uninfected controls at baseline and follow-up. (B-F) Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as unadjusted values. Horizontal bars represent median. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

T cell memory subpopulation analysis

To investigate the effect of schistosome infection on the frequencies of circulating memory T cell subsets we compared peripheral T cells from infected and uninfected children by flow cytometry (Figure 3A). There were no significant differences in total $CD4^+$ T cell levels between schistosome-infected and –uninfected children (34.9% and 37.6% respectively; p=0.508). Naïve and total memory T cells were defined using CD45RA expression. The proportion of memory T cells was significantly higher in infected children at baseline and remained elevated at follow-up; no differences were observed between pre- and post-treatment levels (Figure 3B). Concomitantly levels of naïve T cells were significantly lower in infected children (Figure 3C). Additional staining with CD27 distinguished between central and effector memory T cells resulting in three distinct populations: central memory (T_{CM}) ($CD45RA^-CD27^+$), effector memory (T_{EM}) ($CD45RA^-CD27^-$) and terminally differentiated effector memory cells (T_{EMRA})($CD45RA^+CD27^-$)

[21]. While no differences in the proportion of various subsets were seen at baseline (Figure 3D-F), there was a significant increase in the frequency of effector memory cells (Figure 3E) in the infected children following treatment but not in the uninfected controls.

CD4⁺CD25⁺FOXP3⁺ T cells analysis

We next assessed frequencies of circulating CD4⁺CD25⁺FOXP3⁺ T cells in a subset of infected and uninfected children, at pre- and 7 months post-treatment and also included samples that were available at an additional time point of 6 weeks post-treatment. The subset of children included in this analysis was representative of the whole study population, as no differences

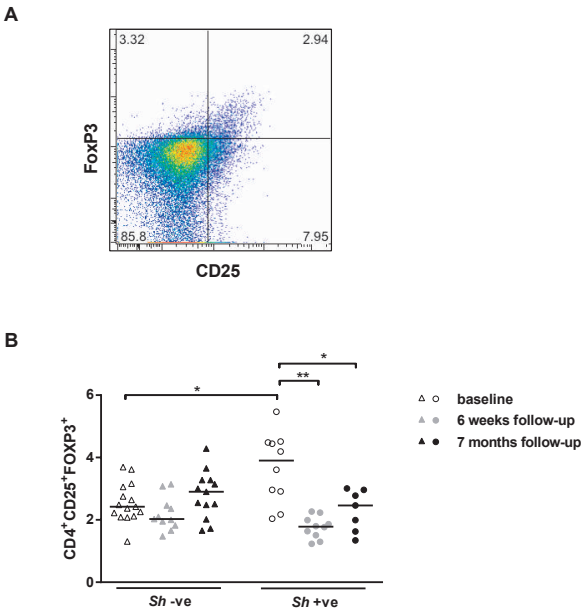

Figure 4: CD4⁺CD25⁺FOXP3⁺ T cell analysis at baseline and 6 weeks and 7 months follow-up.
CD4⁺CD25⁺FoxP3⁺ T cells were identified and enumerated with flow cytometry with initial gating steps performed like in Figure 3 (A) and frequencies determined for *S. haematobium*-infected children and uninfected controls at baseline and at follow-up (B). Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as unadjusted values. Horizontal bars represent median. * p< 0.05, ** p< 0.01, *** p< 0.001.

were found in the characteristics of this subset and the whole population. By analyzing the expression of CD4, CD25 and FOXP3 by flow cytometry (Figure 4A) we showed that the proportion of CD4⁺CD25⁺FOXP3⁺ T cells was significantly higher in infected children at baseline and these frequencies were significantly reduced to levels comparable to the uninfected control group following treatment (Figure 4B). The decrease in CD4⁺CD25⁺FOXP3⁺ T cell levels was more pronounced at 6 weeks compared to 7 months post-treatment (Figure 4B).

Association between $CD4^+CD25^+FOXP3^+$ T cells and cytokine levels in response to schistosomal products

As the decrease in $CD4^+CD25^+FOXP3^+$ T cell levels over time mirrored the increase in cytokine responses to schistosomal antigens in infected children, we further explored this longitudinal association by using linear mixed-effects models (Table 4). We assessed the relationship between $CD4^+CD25^+FOXP3^+$ T cell levels and cytokine response and found that $CD4^+CD25^+FOXP3^+$ T cell levels were significantly and negatively associated with IL-5 and IL-10 levels in response to both SEA and AWA.

Table 4: Association between $CD4^+CD25^+FOXP3^+$ T cells and cytokine levels in response to schistosomal antigens.

		S. haematobium infected children	
Cytokine	**Stimulus**	**Estimate β (95% CI)**	**p value**
IFN-γ	SEA	-0.60 (-1.61 - 0.42)	0.227
	AWA	-0.11 (-0.77 - 0.56)	0.737
IL-5	SEA	-1.04 (-1.91 - -0.18)	0.024
	AWA	-1.15 (-1.69 - -0.62)	0.001
IL-10	SEA	-1.08 (-1.76 - -0.41)	0.005
	AWA	-0.60 (-1.02 - -0.18)	0.010
TNF	SEA	-0.37 (-0.96 - 0.22)	0.188
	AWA	-0.64 (-1.43 - 0.15)	0.102
IL-2	SEA	-0.30 (-1.04 - 0.44)	0.378
	AWA	-0.47 (-0.94 - 0.00)	0.050

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; SEA, schistosomal egg antigen; AWA, adult worm antigen. Parameter estimates are from linear mixed-effects models assessing associations of level of $CD4^+CD25^+FOXP3^+$ T cells and each cytokine/stimulus combination within groups indicated by the subheadings.

Discussion

This is the first study to concurrently examine both innate and adaptive cellular immune responses, T cell memory and levels of $CD4^+CD25^+FOXP3^+$ T cells in schoolchildren infected with *S. haematobium* and to assess the effects of treatment with praziquantel on these responses. Using this approach we show that whereas antigen specific responses are suppressed during infection, this does not extend to innate immune responses. Importantly, we provide evidence that the reduction in $CD4^+CD25^+FOXP3^+$ T cells upon treatment is correlated with increase in effector T cells and antigen specific cytokine production.

In the current study whole blood innate pro-inflammatory (TNF and IL-1β) or anti-inflammatory/regulatory (IL-10 and IL-1ra) cytokine responses in response to TLR stimulation were not affected by current *S. haematobium* infection. This is in contrast to our own previous

studies which showed evidence of altered cytokine response upon TLR activation [9,12,22] and may be due to differences between using whole blood, which contain granulocytes in the current study and isolated PBMCs or cell subsets in our other studies. We also observed that IL-1β and IL-10 in response to Pam3 were lower at the 7 months follow-up time point after treatment in all subjects. This might have resulted from the change in *P. falciparum* infection and/or load which decreased in both the infected and uninfected groups over time and demonstrates why inclusion of appropriate controls, i.e. uninfected controls which are similarly followed over time is important. A prominent decrease in the levels of TNF was observed in the infected group treated with praziquantel; this is in line with the findings that show TNF to be associated with schistosome infection [23,24]. The function of DC-SIGN/MR and Dectin-1 which do not generally induce cytokine responses on their own can be studied by looking at their ability to enhance TLR responses. We did not find any differences between infected and uninfected schoolchildren or between pre- and post-treatment responses in the combined TLR and CLR stimulations.

For adaptive responses anti-schistosome treatment over a 7 month period resulted in enhanced responses to both SEA and AWA. Enhanced cytokine responses at 6-7 weeks post-treatment have been seen in a number of studies before [6–8,25]. Recently, a study examining immune responses at 1- and 2-yr after treatment of Kenyan schoolchildren infected with *S. mansoni*, observed an increase in Th2 responses (IL-4, IL-5, IL-9 and IL-13) to both SEA and AWA, however pro-inflammatory responses (IL-1β, TNF, CCL5 and IL-6) were either decreased in response to SEA or unchanged in response to AWA following treatment [26]. It is interesting to speculate that the decrease in IL-1β, TNF, CCL5 and IL-6 in the Kenyan cohort may represent an innate component of the immune response and may be in line with our own results where a decrease in TNF was observed following treatment. However, it should be noted that the children recruited in the Kenyan study all presented with hepatomegaly and exhibited predominant pro-inflammatory responses at pre-treatment whereas children in our study were free of pathology as assessed by ultrasonography of the bladder (unpublished data). Our current study indicates that the removal of parasites over a longer period of time in children without pathology is associated with sustained enhancement of antigen-specific responses, in agreement with the short-term treatment studies and with the increase in Th2 responses seen in the long-term treatment in the Kenyan study.

Down-regulation of immune responses has been ascribed to a strong immunomodulatory network induced by schistosomes [27]. In this study we show that *S. haematobium* infection is associated with increased frequency of $CD4^+CD25^+FOXP3^+$ T cells and as seen previously for $CD4^+CD25^{hi}$ Tregs in *S. mansoni* infected adults [10] $CD4^+CD25^+FOXP3^+$ T cell numbers decrease after removal of infection. Importantly, the decrease in the level of $CD4^+CD25^+FOXP3^+$ T cells over time following treatment was inversely associated with an increase in IL-5 and IL-10 cytokine production. The source of IL-10 measured here is not clear as cells other than $CD4^+CD25^+FOXP3^+$ T cells are also known to produce IL-10. Moreover this may reflect a general increase of responsiveness leading to more cytokines and possible expansion of schistosome-specific T cell responses as suggested by the increase in effector memory T cells. Future functional studies should address the mechanism by which $CD4^+CD25^+FOXP3^+$ T cells exert

their suppressive function. In addition, $CD4^+$ T cell intrinsic regulation via increased expression of surface inhibitory receptors such as CD200R [28] or increased expression of the E3 ubiquitin ligase GRAIL (gene related to anergy in lymphocytes) [29] may also play a role in observed hypo-responsiveness and likewise warrants further investigation.

When evaluating total memory T cells, we found higher proportions of memory T cells in infected children suggesting that exposure to parasite antigens drives the expansion of the memory T cell pool. This is in contrast to a recent study from Zimbabwe which showed a decrease in $CD4^+$ memory T cells, although this difference was only observed in individuals older than 13 years of age [30]. We specifically observed an increase in the effector memory T (T_{EM}) cells in the infected children following treatment which mirrored the increase in adaptive cytokine production suggesting that hypo-responsiveness may also in part be linked to the memory T cell pool. Whether this hypo-responsiveness is due to decreased cell numbers kept under control by regulatory responses or cell quiescence and/or exhaustion of effector cells, remains to be investigated. In this regard, a recent interesting study of the memory T cell pool in chronic hepatitis infection before and after treatment has shown that inhibitory molecules, PD-1 and CD244, on memory T cells were down-regulated following treatment while effector molecules perforin, granzyme B and IFN-γ were up-regulated resulting in enhanced recovery of memory T cells [31]. Future studies should investigate the phenotype of memory T cells in more depth to further understand how the phenotypic profiles affect the functional capacities of the $CD4^+$ T cells.

In summary, this study shows that infection with *S. haematobium* is associated with antigen-specific hypo-responsiveness, alterations of the T cell memory pool and increased levels of $CD4^+CD25^+FOXP3^+$ T cells and that praziquantel treatment leads to increased effector T cell frequencies and decreased levels of $CD4^+CD25^+FOXP3^+$ T cells which was associated with increased antigen-specific cytokine production.

Materials and methods

Ethics statement

The study was approved by the "Comité d'Ethique Régional Indépendent de Lambaréné" (CERIL; N°06/08). Written informed consent was obtained from parents or legal guardians of all schoolchildren participating in the study.

Study population and parasitology

Between November 2008 and February 2009, 100 schoolchildren were invited to participate in a longitudinal study to investigate the effect of *S. haematobium* infection on innate and adaptive immune responses. Children were recruited from a rural area surrounding Lambaréné (Gabon) where *S. haematobium* infection is endemic as previously described [12]. To be included in the study, participants had to meet the following criteria: (1) attendance at the local school; (2) no praziquantel treatment in the previous year; (3) no fever (body temperature < 37.5°C) and (4) no other symptoms of acute illness. Seventy-nine schoolchildren returned for follow-up performed 7 months later between June and September 2009. There were no significant

differences between the schoolchildren that returned for follow-up and those that did not.

S. haematobium infection was determined prior to blood collection by examining a filtrate of 10 mL of urine passed through a 12-µm-poresize filter (Millipore). Children were classified *S. haematobium*-infected if at least one *S. haematobium* egg was detected in the urine, or uninfected if three consecutive urine samples were negative. Infections with intestinal helminths *Ascaris lumbricoides, Trichuris trichiura* and hookworm were determined by analyzing one fresh stool sample using the Kato-Katz method [18]. Infection with *Plasmodium falciparum* was determined by PCR [19] with a cut-off value of 35 Ct. After collection of blood samples, all *S. haematobium*-infected children were treated with a single dose of praziquantel (40 mg/kg) three times every two months. Intestinal helminth- and malaria-infected children received respectively a single dose of albendazole (400 mg) or an artemisinin-based combination therapy as per the local guidelines.

Hematological parameters were analyzed using ADVIA 120 Hematology System (Bayer Health Care) and erythrocyte sedimentation rate was determined manually.

Circulating anodic antigen (CAA) testing of plasma samples was performed using the upconverting phosphor (UCP) technology lateral flow assay as previously described [20].

Reagent strips (Cobas) were used to assess hematuria, leukocyturia and proteinuria. Individual scores for each of these parameters, ranging from 0 to 3, were added together for calculation of the combined reagent strip index (RSI).

Whole blood culture

Five hours following venipuncture, heparinized blood was diluted 2 times with RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin (Astellas), 10 µg/mL streptomycin, 1 mM pyruvate and 2 mM L-glutamine (all from Sigma-Aldrich). To assess adaptive responses whole blood was incubated for 72 hours with *S. haematobium* soluble egg antigen (SEA) or adult worm antigen (AWA) (both 10 µg/mL) or 2 µg/mL PHA (Remel). To assess innate responses whole blood was incubated for 24 hours with 100 ng/mL Pam3CSK-SKKKK (EMC Microcollection), 100 µg/mL mannan (Sigma-Aldrich) or 100 µg/mL curdlan (prepared in 15mM NaOH; Wako Chemicals) alone or in combination. Medium without stimulus was used as a negative control. 100 µL of ligand(s) in medium was added to wells containing 100 µL of diluted blood in 96-well round bottom plates (Nunc) and incubated at 37°C in the presence of 5% CO_2. Supernatants were stored at -80°C.

Cytokine analysis

Supernatants were analyzed simultaneously for IFN-γ, IL-2, IL-5, IL-10 and TNF or IL-10, IL-1β, IL-1ra and TNF using customized Luminex cytokine kits (Invitrogen) according to manufacturers' recommendations. Samples were acquired with Luminex-100 cytometer (Luminex Corporation). Samples with concentrations below the detection limit were assigned values corresponding to half of the lowest value detected. For the IFN-γ, IL-2, IL-5, IL-10 and TNF multiplex assay the lowest values detected were 0.090 pg/mL for IFN-γ, 0.063 pg/mL for IL-2, 0.044 pg/mL for IL-5, 0.063 pg/mL for IL-10, and 0.051 pg/mL for TNF. For the IL-10, IL-1β, IL-1ra and TNF multiplex assay the lowest values detected were 0.016 pg/mL for IL-10, 0.255 pg/mL for IL-1β, 2.927 pg/

mL for IL-1RA and 0.016 pg/m for TNF.

Characterization of memory and CD4$^+$CD25$^+$FOXP3$^+$ T cells in peripheral blood

For immunophenotyping memory T cells freshly isolated PBMCs were fixed in 2.4% formaldehyde solution (Sigma-Aldrich) for 15 minutes at room temperature and, subsequently, frozen in RPMI 1640 medium supplemented with 20% fetal bovine serum (Greiner Bio-One) and 10% dimethyl sulfoxide (Merck) and stored at −80°C until flow cytometric analysis. After thawing, cells were washed and stained for 30 minutes with anti-CD4-PE-Cy7 (RPA-T4; eBioscience), anti-CD45RA-Horizon V450 (HI100; BD Biosciences) and anti-CD27-FITC (L128; BD Biosciences). To assess levels of CD4$^+$CD25$^+$FOXP3$^+$ T cells, ex-vivo PBMC were fixed with the FOXP3 fixation/permeabilization kit (eBisocience) and stored as described above. After thawing cells were stained with CD4-PE-Cy7 (SK3; BD Biosciences), CD25-PE (2A3; BD Biosciences) and FOXP3-APC (PCH101; eBioscience). Cells were acquired on FACSCanto II flow cytometer (BD Biosciences) and analyzed in FlowJo software v9 (Tree Star Inc.); Boolean gates were used for memory T cell analysis.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 20.

Differences between the study groups for gender, malaria and intestinal helminth infections were tested using Fisher's exact test. Differences in malaria prevalence pre- to post-treatment were tested with the McNemar's test. Age, erythrocyte sedimentation rate (ESR) and hemogram parameters were normally distributed and differences between study groups were tested using the independent student's T test. Infection indices, including schistosome egg counts, plasma CAA levels, hematuria and RSI scores and T cell subset differences were compared with the Mann-Whitney U test. Within group differences between baseline and follow-up were compared with the Wilcoxon matched pairs test. Correlations between infection indices were analyzed by Spearman's rank correlation.

Cytokine concentrations in response to stimulation were corrected for spontaneous cytokine production by subtracting responses of unstimulated medium wells to obtain net cytokine responses, with negatives values set to half of the lowest value detected per given cytokine. Net cytokine responses were normalized by log(base 10)-transformation after adding 1 pg/mL.

Interaction ratio between combined TLR and CLR stimulation was calculated by dividing net cytokine response to PAM3 in combination with mannan or curdlan by the sum of net cytokine responses obtained with each ligand alone i.e. [PAM3 + mannan]/([PAM3] + [mannan]) or [PAM3 + curdlan]/([PAM3] + [curdlan]). The interaction ratio could result in a synergistic or inhibitory effect. A paired samples T test was used to test whether the resulting interaction was significant by comparing combined stimulation with the sum of cytokines produced in two separate stimulations.

Univariate GLM analysis adjusted for age was used to compare between infection groups and the resulting adjusted means were anti-log-transformed for graphing purposes. Differences within the same group between baseline and follow-up were compared by paired samples T

test.

Linear mixed-effects model was used to assess the longitudinal association between $CD4^+CD25^+FOXP3^+$ T cell levels and cytokine responses to schistosomal products with cytokine levels as the dependent variable; participant ID as a random effect; infection group as a fixed effect; and $CD4^+CD25^+FOXP3^+$ T cell level as the covariate. The variance–covariance structure "Toeplitz" was used.

For all tests, statistical significance was considered at the 5% level. To adjust for multiple comparisons, where applicable, Bonferroni-Holm correction was taken into account.

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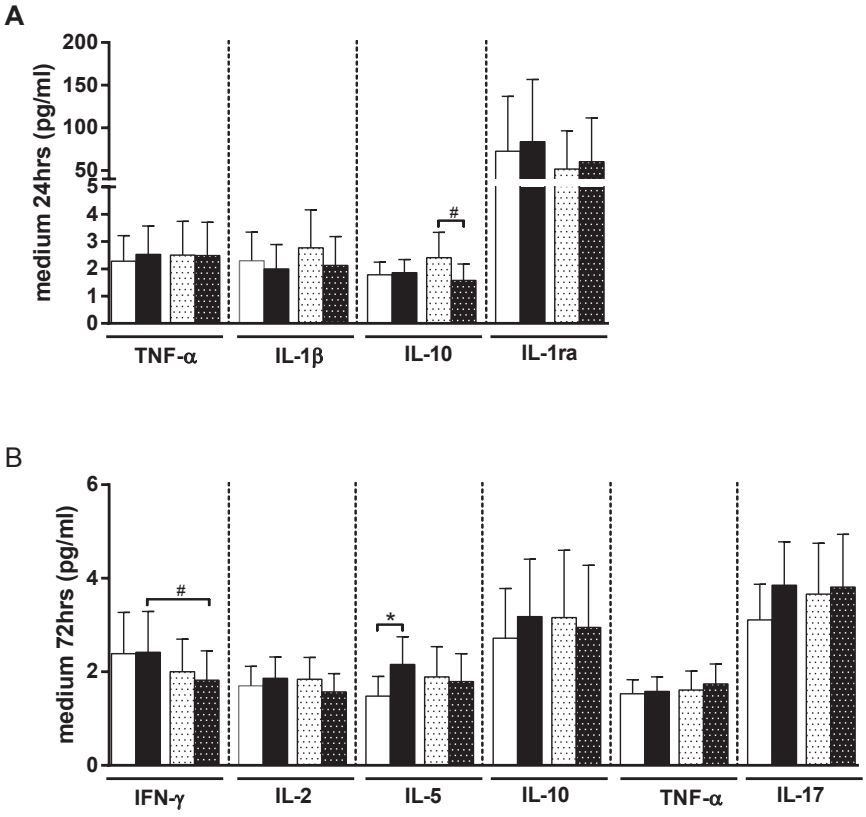
Supplementary material



Supplementary Figure 1: Cytokine production in 24hr and 72hr unstimulated whole blood cultures at baseline and 7 months follow-up.

Cytokine levels were measured in 24hr unstimulated cultures (A) by Luminex (IL-5, IFN-γ, IL-2, IL-10 and TNF). Cytokine levels were measured in 72hr unstimulated cultures (B) by Luminex (TNF, IL-1β, IL-10 and IL-1ra). Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as age-adjusted geometric means.

Supplementary table 1: Interaction between TLR and non-TLR ligands.

		S. haematobium uninfected		*S. haematobium* infected	
Cytokine	**Stimulus combination**	**Interaction ratio (95% CI)**	**p value**	**Interaction ratio (95% CI)**	**p value**
TNF	Pam3 + mannan	1.86 (1.19 - 2.91)	0.005	1.43 (0.90 - 2.25)	0.016
	Pam3 + curdlan	16.22 (9.89 - 26.60)	<0.001	10.76 (6.57 - 17.67)	<0.001
IL-1β	Pam3 + mannan	2.69 (2.29 - 3.15)	<0.001	2.48 (2.12 - 2.90)	<0.001
	Pam3 + curdlan	7.96 (6.26 - 10.15)	<0.001	9.79 (7.71 - 12.42)	<0.001
IL-10	Pam3 + mannan	1.29 (1.15 - 1.45)	<0.001	1.26 (1.13 - 1.42)	<0.001
	Pam3 + curdlan	0.73 (0.60 - 0.88)	<0.001	0.71 (0.59 - 0.86)	0.002
IL--1ra	Pam3 + mannan	1.15 (1.06 - 1.25)	<0.001	1.06 (.98 - 1.15)	0.225
	Pam3 + curdlan	0.59 (0.52 - 0.67)	<0.001	0.54 (0.48 - 0.61)	<0.001

TNF, tumor necrosis factor; IL, interleukin. Interaction ratio: [PAM3 + mannan]/([PAM3] + [mannan]) or [PAM3 + curdlan]/([PAM3] + [curdlan]). A paired samples T test was used to test whether the resulting interaction was significant by comparing combined stimulation with the sum of cytokines produced in two separate stimulations. All p values below 0.05 remained significant after α-adjustment by the Bonferroni-Holm method.