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Pronounced CD4^{+}CD25^{hi}FOXP3^{+} regulatory T cell activity in human schistosomiasis before and after treatment with praziquantel.

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Abstract

Human schistosomiasis, one of the most common parasitic infections worldwide, survives within its immunocompetent host for several years. This is thought to be due to its ability to down-regulate host immune responses. In this study we examined the effect of $CD4^{+}CD25^{hi}FOXP3^{+}$ regulatory T cells (Tregs) on cell proliferation and cytokine production in response to schistosome egg (SEA) and adult worm (AWA) antigens and to the vaccine-antigen Bacillus Calmette–Guérin (BCG). These responses were studied in peripheral blood mononuclear cells (PBMC) of children infected with *Schistosoma haematobium* before and 6 weeks after anthelmintic treatment with praziquantel. Schistosome infection was associated with increased frequencies of Treg cells, which decreased following treatment. The measured cytokine (IFN-γ, IL-5, IL-10, IL-13, IL-17 and TNF) data was integrated using Principal Component Analysis (PCA). The cytokines clustered into two principal components, with principal component 1 (PC1) reflecting regulatory and Th2-polarized cytokine responses and principal component 2 (PC2) reflecting pro-inflammatory and Th1-polarized cytokine responses. The reduction in Treg frequencies after treatment was accompanied by an increase in both PC1 and PC2 following treatment compared to pre-treatment values. Proliferation, measured by CFSE dilution of dividing cells, showed no significant difference from pre- to post-treatment. To specifically assess Treg function, PBMC responses to antigens was measured after Treg depletion. Treg depletion resulted in increased values of both PC1 and PC2 at both pre-treatment and also at 6 weeks post-treatment. Proliferative responses were for the most part only significantly affected by Treg depletion in infected individuals at pre-treatment. These results show that $CD4^{+}CD25^{+}FOXP3^{+}$ T cells are higher in *S. haematobium*-infected subjects and decrease after treatment. Functionally, their suppressive activity on cytokine production does not change after treatment but their ability to influence proliferation weakens with treatment.

Introduction

The immune system has evolved several regulatory mechanisms to maintain immune homeostasis, prevent autoimmunity and restrain inflammation [1–3]. Many pathogens have developed mechanisms to manipulate the regulatory network of the host to their advantage, thereby generating conditions that ensure their survival for a prolonged period of time. In particular FOXP3$^+$ regulatory T cells (Tregs) have been shown to play a major role in the control of various parasitic infections suppressing local tissue damage and pathology that would result from otherwise over-reactivity. However, enhanced Treg cell activity may also allow the long-term survival of the parasite as the host is hampered from fighting the intruding pathogen effectively [4].

Schistosomiasis is a helminth infection affecting over 240 million people worldwide, especially children [5]. When chronic in nature it has been shown to be associated with general T cell hypo-responsiveness – evident from down-modulated antigen-specific Th1 and Th2 cell responses [6,7]. This might results from mechanisms involving peripheral anergy and suppression triggered by regulatory cells, such as Tregs [8]. For example, in experimental murine models, it was observed that the presence of Tregs suppressed the development of pathology [9], and down-modulated adequate Th1 and Th2 responses [10,11], promoting parasite survival within the host [12,13]. Evidence for Treg cell activity in human helminth infections has been provided by the detection of T cells with a regulatory phenotype in lymphatic filariasis- [14], in onchocerciasis- [15,16] and in schistosomiasis patients [17].

The killing of the schistosome parasites using effective chemotherapy with praziquantel (PZQ) has previously been shown to result in elevated Ag-specific proliferation and cytokine production, in particular IL-4, IL-5, and IFN-γ [6,18–21]. Although, the frequency of Treg cells, defined phenotypically as CD4$^+$CD25^{hi}FOXP3$^+$, appears to decrease substantially after treatment with praziquantel [17], their functional activity has not been studied before. One previous study established a positive correlation between Treg proportion and infection intensity only in children up to the age of 14, while in children > 14 years of age the opposite was the case [22].

Few studies have investigated the frequency and functional capacity of regulatory T cells on responses to helminth and vaccine antigens in infected children not only before but also after anthelmintic treatment. In the current longitudinal study we enumerated regulatory T cells in peripheral blood of *S. haematobium*-infected children at baseline and 6 weeks after treatment. We also evaluated proliferative and cytokine responses in total and Treg-depleted PBMC, both at pre- and post-treatment in order to determine the functional activity and capacity of Tregs in presence and absence of infection.

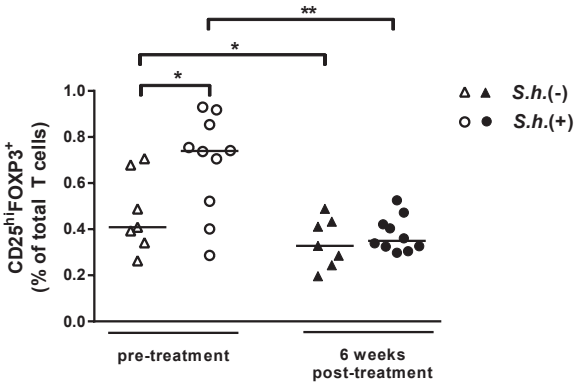
Results

Elevated levels of CD4$^+$CD25^{hi}FOXP3$^+$ Treg cells in *S. haematobium*-infected schoolchildren

To investigate whether *Schistosoma haematobium* infection affects the frequency of peripheral blood Treg cells we compared circulating CD4$^+$CD25^{hi}FOXP3$^+$ Treg cells from infected and

Figure 1: Increased frequency of CD25^{hi}FOXP3^{+} Treg cells during *S. haematobium* infection.

CD4 T cells were identified and Boolean gating combinations were used to determine proportions of CD4^{+}CD25^{hi}FOXP3^{+} Treg cells. Differences between groups were tested with a Mann-Whitney U test and within groups with a Wilcoxon matched pairs test. Horizontal bars represent median. * p< 0.05, ** p< 0.01.



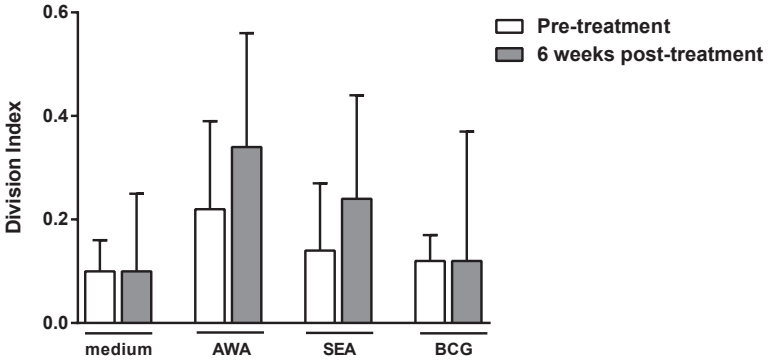


Figure 2: Proliferative responses to schistosome specific and non-specific antigens.

CFSE-labeled PBMC pre- and 6 weeks post-treatment were left unstimulated (medium), or stimulated with *S. haematobium* adult worm antigen (AWA) and soluble egg antigen (SEA) and Bacillus Calmette–Guérin (BCG). After 4 days of culture cells were fixed, cryopreserved and after thawing CFSE division was analyzed by flow cytometry. Results are shown as median with IQR. Differences between pre-treatment and 6 weeks post-treatment responses were tested with a Wilcoxon matched pairs test.

Table 1: Description of Principal Components.

	Principal Component	
	1	**2**
IL-5	**0.897**	-0.250
IL-10	**0.774**	0.076
IL-13	**0.926**	-0.129
IL-17	0.080	**0.664**
IFN-γ	-0.351	**0.853**
TNF	-0.098	**0.875**

Arbitrary values indicate the relative loading of each cytokine response towards each principal component. Strong positive loadings (>0.500) are indicated in bold. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

uninfected children by flow cytometry. We found that frequencies of $CD4^{+}CD25^{hi}FOXP3^{+}$ Treg cells were significantly higher in infected children compared to uninfected children (Figure 1). Importantly, 6 weeks after praziquantel treatment Treg frequencies were significantly reduced to levels comparable to the uninfected control group. Treg frequencies in the uninfected group were also reduced over the same period although much less so.

Proliferation and cytokine production in response to schistosome-specific and non-specific antigens in *S. haematobium*-infected schoolchildren at pre-treatment and 6 weeks post-treatment

Next, we assessed the effect of anthelmintic treatment on cell proliferation and cytokine production in response to stimulation with schistosome-specific antigens SEA and AWA and a non-specific antigen BCG; raw cytokine values are shown in supplementary table 2. Proliferation was determined by calculating the division index on the basis of the dilution of CFSE in PBMCs. There were no significant differences in proliferation between pre-treatment and 6 weeks post-treatment responses (Figure 2). Cytokine production on the other hand significantly changed between pre-treatment and 6 weeks post-treatment. We applied Principle Component Analysis (PCA) in order to provide a more global assessment of the effect of schistosome infection on responses to not only SEA and AWA stimulation but also to a third-party antigen BCG. PCA converts a large dataset of variables into a set of summary variables called principal components which represent variables that share a high level of correlation. Two distinct principal components were identified: principle component 1 (PC1) which reflects regulatory and Th2-polarized cytokine responses due to its positive loading with IL-5, IL-10 and IL-13 responses; and principle component 2 (PC2) which reflects pro-inflammatory and Th1-polarized cytokine responses due to its positive loading with IFN-γ, IL-17 and TNF (Figure 3, Table 1 and Table 2). We saw a significant increase in both PC1 and PC2 following treatment compared to baseline values (Table 3).

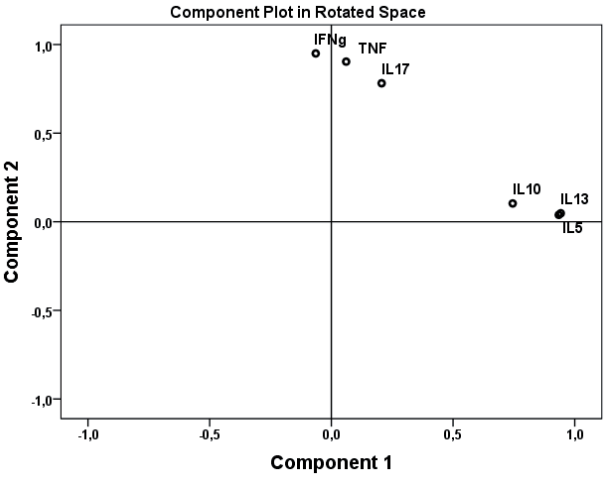


Figure 3: Principal component analysis (PCA) of cytokine responses to schistosome specific and non-specific antigens.
Two distinct principal components were identified: principle component 1 (PC1) which reflects regulatory and Th2-polarized cytokine responses due to its positive loading with IL-5, IL-10 and IL-13 responses; and principle component 2 (PC2) which reflects pro-inflammatory and Th1-polarized cytokine responses due to its positive loading with IFN-γ, IL-17 and TNF.

Table 2: Variance explained by the principal components extracted from the cytokine data.

	Initial Eigenvalues		
Component	**Total**	**% of variance**	**Cumulative %**
1	2.86	40.01	40.01
2	1.56	33.65	73.66

Two principal components were extracted from the data. The percentage of the data explained by each of the components individually and cumulatively is shown.

Table 3: Changes in cytokine production in response to antigens pre- to post-treatment.

Component	Time-point	Mean Rank	p value
1	pre-treatment	27.04	0.004
	post-treatment	41.14	
2	pre-treatment	31.41	0.021
	post-treatment	38.07	

Enhanced T cell proliferation in PBMC from schistosome-infected children after Treg depletion

To study the suppressive effect of Treg cells on proliferation and cytokine responses, $CD4^+CD25^{hi}FOXP3^+$ T cells were depleted from PBMC by magnetic beads. The $CD4^+CD25^{hi}FOXP3^+$ population decreased by 60%, $p < 0.0001$; a representative example is shown in Figure 4.

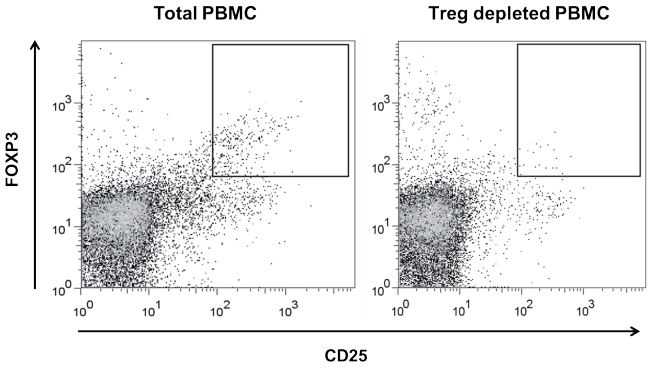


Figure 4: Treg depletion. $CD4^+CD25^{hi}$ T cells were depleted by magnetic bead separation. Representative examples of the depletion of the $CD4^+CD25^{hi}FOXP3^+$ population.

Depletion of Treg cells at pre-treatment resulted in enhanced spontaneous proliferation (medium condition) as well as in enhanced proliferation to specific schistosomal antigens AWA and SEA and to vaccine antigen BCG (Figure 5A). Interestingly, at 6 weeks after anthelmintic treatment Treg depletion resulted in significant increase in proliferation in response to AWA only. A typical plot of CFSE staining showing the effect induced by depletion of Treg cells (Figure 5B).

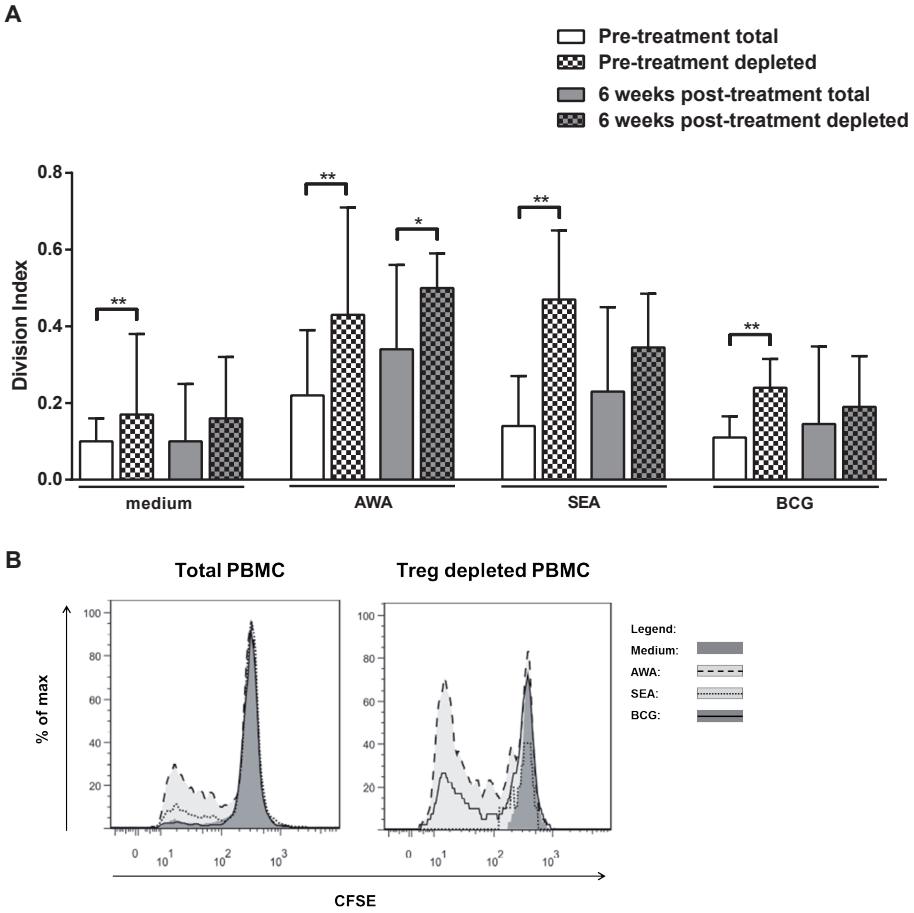


Figure 5: Effect of Treg depletion on proliferative responses to schistosome specific and non-specific antigens.

CFSE-labeled total or $CD4^+CD25^{hi}FOXP3^+$ depleted PBMC pre- and 6 weeks post-treatment were left unstimulated (medium), or stimulated with *S. haematobium* adult worm antigen (AWA) and soluble egg antigen (SEA) and Bacillus Calmette–Guérin (BCG). After 4 days of culture cells were fixed, cryopreserved and after thawing CFSE division was analyzed by flow cytometry. Results are shown as median with IQR (A). Differences between total and depleted PBMC were tested with a Wilcoxon matched pairs test. * $p< 0.05$, ** $p< 0.01$. Representative plot of CFSE staining illustrating proliferation of total or Treg depleted PBMC (B).

Increased cytokine responses following Treg depletion

Next, we investigated the capacity of Tregs to suppress cytokine responses by evaluating the effect of $CD4^+CD25^+FOXP3^+$ T cell depletion on principal component 1 (IL-5, IL-10 and IL-13) and principal component 2 (IFN-γ, IL-17 and TNF). We found $CD4^+CD25^+FOXP3^+$ T cell depletion at pre-treatment resulted in increased values of both PC1 and PC2 in infected individuals, and similarly following treatment we also observed an increase in the values of both PC1 and PC2 in the now infection free schoolchildren (Table 4).

Table 5: Changes in cytokine production in response to antigens in total and Treg depleted PBMC at pre- and post-treatment.

Component	Time-point	Total/ Treg depleted PBMCs	Mean Rank	p value
1	pre-tx	total	32.78	0.002
		depleted	42.36	
	post-tx	total	33.58	0.002
		depleted	39.38	
2	pre-tx	total	28.09	<0.001
		depleted	43.36	
	post-tx	total	32.35	<0.001
		depleted	39.41	

tx, treatment.

Discussion

Down-regulation of immune responses has been attributed to a strong immunomodulatory network induced by schistosomes and is mediated most prominently by regulatory T cells [23]. Here we demonstrate that $CD4^+CD25^+FOXP3^+$ Treg cells are increased during *S. haematobium* infection and their numbers decrease after removal of infection with praziquantel. A much smaller, yet statistically significant decrease was also observed in the frequency of these cells in the control uninfected group. This change shows the importance of including controls that could indicate any technical or environmental changes that might be associated with longitudinal studies.

In order to obtain a global assessment of the effect of *S. haematobium* infection on Th1, Th2, regulatory and pro-inflammatory cytokine responses we applied PCA analysis. This allowed us to summarize the various responses into two principal components [24]. Principle component 1 (PC1) reflected regulatory and Th2-polarized cytokine responses due to its positive loading with IL-5, IL-10 and IL-13, responses commonly associated with chronic schistosome infections. Principle component 2 (PC2) reflected pro-inflammatory and Th1-polarized cytokine responses due to its positive loading with IFN-γ, IL-17 and TNF, responses more commonly associated with acute schistosome infection or bacterial infections such as tuberculosis. We show that *S. haematobium* infection is associated with hypo-responsiveness as demonstrated by increases in cytokine production represented by both PC1 and PC2. T cell division was also assessed, but despite the consistently higher proliferation to all stimuli tested, at post treatment, the change was not statistically significant. These data indicate that the increased frequency of $CD4^+CD25^+FOXP3^+$ Tregs during schistosome infection is associated with poor cytokine responsiveness.

To assess the functional capacity of the regulatory T cells, a field applicable method was used which consists of the depletion of regulatory T cells from PBMC to assess their effect on cytokine production or proliferation. The data show that depletion of Tregs is associated with increased cytokine production, of both PC1 and PC2 which means that both Th2/regulatory

and Th1/pro-inflammatory cytokine production improves. This is the case at both pre-treatment and post-treatment time points, although the increase appears to be stronger at pre-treatment. Altogether, this would suggest that even though regulatory T cell numbers change with infection, their functional capacity to induce suppression is not affected as profoundly.

We furthermore evaluated the effect of Treg depletion on cell proliferation and in contrast to cytokine responses which were equally affected at pre-and post-treatment, proliferative responses were for the most part only significantly affected by Treg depletion in infected individuals at pre-treatment. These data could be explained if the ability of Treg to suppress proliferation would need less profound or distinct suppressive mechanisms than what is required for inhibiting cytokine production. The removal of *S. haematobium* infection could then affect the Treg function partially. Tregs are thought to exert their function via a number of different mechanism including IL-10 and/or TGF-β production, IL-2 consumption, or cell-cell contact where inhibitory molecules such as CTLA-4 and PD-1 are key [25,26]. Future studies are needed to further delineate how Treg cells exert their suppressive role during the course of schistosome infection and furthermore the difference in mechanism between suppression of proliferation versus effector cytokine production. Additional alternative mechanism, such as T cell anergy due to increased expression of the E3 ubiquitin ligase GRAIL (gene related to anergy in lymphocytes) which has been shown in a mouse model to be linked to Th2 cell hypo-responsiveness should also be investigate [27].

Alternatively, recently described regulatory $CD8^+$ T cells which likewise produce IL-10, may also in part contribute to the differences observed [28,29]. $CD25^+$ cell depletion will in addition to depleting $CD4^+CD25^+FOXP3^+$ T cells also deplete the $CD8^+CD25^+FOXP3^+$ T cell population and therefore future studies are needed to re-assess the relative contributions of these different subsets. Moreover studies with more extensive panels of markers associated with suppressive T cell functions are necessary as FOXP3 expression has been shown to be transiently up-regulated on activated $CD4^+$ T cells,

In summary, this study shows that infection with *S. haematobium* is associated with alterations of the frequency and activity of $CD4^+CD25^{hi}FOXP3^+$ regulatory T cells and that these in turn affect proliferation and global cytokine responses. These data indicate that functional activity of regulatory T cells needs to be taken into consideration when studies consider coinfections, treatment or vaccine responses in areas where helminths are prevalent.

Materials and methods

Study population

Study participants were schoolchildren attending the school of PK15, approximately 15 km south of Lambaréné in the province of Moyen-Ogouée, Gabon. The area is known to be endemic for *Schistosoma haematobium* (Table 5) (28),(33). The ethical approval for the study was obtained from the Comité d' Ethique Régional Indépendant de Lambaréné (CERIL). Prior to inclusion of study participants, signed consent was obtained from parents or legal guardians. The study included 28 schoolchildren with at least one *S. haematobium* egg in the urine sample when enrolled. Exclusion criteria were (1.) anthelmintic treatment within the last 6 weeks prior

Table 5: Characteristics of the study population.

	Pre-treatment	6 weeks post-treatment
Participants (n)	28	28
Mean age in years (SD)	10.32 (2.2)	n/a
Sex (female/male)	15/13	n/a
Egg count/10 ml urine: median (IQR)	72.5 (24.5 – 296.3)	0.0 (0.0 – 1.5)
Level of haemoglobin (g/dL): mean (SD)	11.13 (0.97)	11.09 (0.94)
Level of white blood cell (x103/µl): mean (SD)	8.76 (3.18)	9.44 (2.92)

to the study, (2.) the presence of fever (tympanic body temperature > 37.5ºC) and/or (3.) others symptoms of acute illness. To compare the frequency of $CD25^{hi}FOXP3^{+}$ Treg cells between *S. haematobium* infected and uninfected schoolchildren an additional 17 participants were recruited (Supplementary table 1); 10 *S. haematobium* infected participants were recruited from PK15 and 7 uninfected participants were recruited from Lambaréné.

Detection of *S. haematobium* infection

A midstream urine sample was collected between 10:00 and 14:00 hours to coincide with the peak of *S. haematobium* egg excretion in urine (34). Infection was determined by passing 10 ml of urine through a 12.0 µm polyamide N filter (Millipore) for the detection of *S. haematobium* eggs (14).

Praziquantel treatment

S. haematobium infected schoolchildren were treated with a single dose of PZQ (40 mg/kg). Three weeks later, in order to ensure clearance of parasites, the same treatment was administered a 2nd time. Six weeks after the first treatment efficacy of PZQ was assessed by measuring the egg load in urine. Donors remaining egg-positive after the 2nd treatment were given a third dose of PZQ and excluded from analysis, if their reduction in egg count was less than 90% (N=5).

Cell isolation and depletion

Peripheral blood mononuclear cells (PBMCs) were purified from heparinized venous blood (7-10ml) by Ficoll-Hypaque centrifugation (Amersham Biosciences, Netherlands). Depletion of $CD25^{hi}$ T cells was performed using a suboptimal concentration of CD25 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

PBMC culture for proliferation and cytokine production

To analyse proliferation green-fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich, CA, USA) was used; CFSE divides over daughter cells upon cell division and can be tracked by decreasing fluorescence intensity. $CD25^{hi}$ depleted and total PBMC were stained with 2 µM CFSE for 15 minutes at room temperature prior to culture. After labelling, cells were cultured in RPMI 1640 (Gibco, Invitrogen®, Carlsbad, CA, USA), supplemented with 10% FCS

(Greiner Bio-One GmbH, Frickenhausen, Germany), 100 U/ml penicillin (Astellas, Tokyo, Japan), 10 µg/ml streptomycin, 1mM pyruvate and 2mM L-glutamine (all from Sigma-Aldrich, CA, USA). Cells were stimulated in round-bottom plates with medium, 10 µg/ml AWA, 10 µg/ml SEA or 10 µg/ml BCG and incubated in the presence of 5% CO_2 at 37.5°C. After 4 days, supernatants were collected and stored at -80°C, while cells were harvested, fixed with 2% formaldehyde (Sigma-Aldrich, CA, USA) and, subsequently, frozen in RPMI 1640 medium supplemented with 20% FCS and 10% DMSO (Merck KGaA, Darmstadt, Germany) and stored at -80°C.

Flow cytometry analysis (FACS)

After thawing, CFSE-labelled cells were incubated with CD4-PE and CD25-APC (all from BD Bioscience, San Diego, CA, USA), acquired on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) and data were analysed in a FlowJo Proliferation application (Tree Star Inc., Ashland, OR, US) by calculation of the fraction of cells that had divided from the starting population (division index). To assess levels of $CD25^{hi}FOXP3^{+}$ Treg cells, ex-vivo PBMC were fixed with the FoxP3 fixation/permeabilization kit (eBisocience, San Diego, CA, USA) and frozen in RPMI 1640 medium supplemented with 20% FBS and 10% DMSO and stored at -80°C. For immunophenotyping isolated PBMCs were stained with CD4-PE/Cy7 (SK3; BD Biosciences, San Diego, CA, USA), CD25-PE (2A3; BD Biosciences, San Diego, CA, USA) and FOXP3-APC (PCH101; eBioscience, San Diego, CA, USA), cells were acquired on FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA) and analysed in FlowJo software (Tree Star Inc., Ashland, OR, US) using Boolean combination gates.

Cytokines assays

Cytokines were measured from supernatants using Luminex 100 IS System (Invitrogen, Carlsbad, CA, USA) and commercially available beads and standards from BioSource (Bleiswijk, Netherlands) for interferon-gamma (IFN-γ), interleukin-5 (IL-5), IL-10, IL-13 and IL-17 and tumor necrosis factor (TNF). Beads were titrated for optimal dilution and used according to manufacturer's instructions.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 20.

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.

To avoid type I and type II errors in multiple testing, immunological parameters were reduced by principal-components analysis (PCA). First, R v2.15.1 Development Core Team software (R Foundation for Statistical Computing, Vienna, Austria, 2012, http://www.R-project.org) was used to estimate Box-Cox transformation parameter for each cytokine to increase normality of the data. Principal Component Analysis with Varimax rotation was used to reduce the data into a smaller number of uncorrelated variables. Rotation converged in 3 iterations. Principal components (PC) with eigenvalues greater than 1 were selected, and PC scores were

computed for each combination of conditions: stimuli AWA/SEA/BCG; total and Treg depleted PBMC; pre- and post-treatment.

Differences between pre- and post-treatment and Treg depleted and total PBMC were tested with the Wilcoxon matched pairs test. For all tests, statistical significance was considered at the 5% level.

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Supplementary material

Supplementary table 1: Characteristics of the study population for assessment of $CD25^{hi}FOXP3^{+}$ Treg cells.

	***S.haematobium* uninfected**	***S.haematobium* infected**
Participants (n)	7	10
Mean age in years (SD)	12.9 (2.6)	12.5 (1.5)
Sex (female/male)	4/3	9/1
Egg count/10 ml urine: median (IQR)	0	19.5 (3.25 – 216.5)
Level of haemoglobin (g/dL): mean (SD)	10.86 (0.72)	11.4 (0.31)
Level of white blood cell ($x10^3$/µl): mean (SD)	6.33 (0.73)	8.82 (0.55)

Supplementary table 2: Raw cytokine values following antigen stimulation in total and Treg depleted PBMC at pre- and post-treatment.
IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; AWA, adult worm antigen; SEA, soluble egg antigen; BCG, Bacillus Calmette–Guérin (BCG)

		Pre-treatment		6 weeks post-treatment	
Cytokine	**Stimulus**	**Total (median (IQR))**	**Treg depleted (median (IQR))**	**Total (median (IQR))**	**Treg depleted (median (IQR))**
IL-5	medium	28.97 (10.61 - 91.81)	29.67 (9.75 - 90.39)	69.40 (39.08 - 154.05)	43.31 (16.58 - 149.90)
	AWA	475.86 (173.95 - 774.07)	372.44 (166.76 - 827.32)	721.88 (373.64 - 1185.76)	745.63 (337.48 - 1112.00)
	SEA	127.50 (62.61 - 241.38)	156.14 (115.71 - 615.18)	184.83 (89.09 - 469.41)	257.78 (102.46 - 440.22)
	BCG	4.96 (3.00 - 13.63)	4.48 (3.00 - 14.75)	14.11 (3.00 - 36.76)	12.67 (3.23 - 38.88)
IL-13	medium	101.43 (14.34 - 141.82)	88.75 (33.64 - 198.27)	142.17 (47.78 - 341.57)	163.38 (47.78 - 363.11)
	AWA	570.86 (339.74 - 1155.67)	798.86 (484.03 - 1237.20)	1047.52 (657.20 - 1984.41)	1359.63 (714.84 - 2126.23)
	SEA	254.60 (135.16 - 467.21)	442.24 (280.61 - 946.45)	522.26 (150.21 - 897.83)	728.83 (277.69 - 1357.21)
	BCG	32.70 (10.06 - 82.64)	85.59 (18.02 - 146.92)	83.64 (17.36 - 217.83)	98.46 (28.14 - 255.57)
IFN-γ	medium	10.01 (5.00 - 28.82)	28.82 (14.34 - 58.51)	11.37 (5.00 - 30.13)	13.36 (5.00 - 66.35)
	AWA	13.52 (5.00 - 36.19)	43.64 (12.02 - 106.81)	6.98 (5.00 - 30.10)	26.69 (5.53 - 91.46)
	SEA	8.18 (5.00 - 24.08)	30.03 (10.25 - 102.82)	6.95 (5.00 - 29.77)	41.74 (9.56 - 75.56)
	BCG	266.25 (84.88 - 911.19)	728.69 (400.98 - 2457.31)	377.74 (200.41 - 1466.34)	1262.53 (453.56 - 3996.58)
IL-10	medium	15.00 (15.00 - 28.61)	15.00 (15.00 - 17.63)	15.00 (15.00 - 42.94)	15.00 (15.00 - 32.71)
	AWA	199.99 (92.99 - 306.86)	199.40 (110.98 - 387.70)	288.56 (120.15 - 564.33)	444.80 (212.37 - 645.22)
	SEA	132.81 (65.02 - 216.76)	164.55 (84.82 - 282.93)	186.01 (77.29 - 373.99)	244.98 (86.71 - 348.82)
	BCG	63.48 (15.00 - 117.97)	63.07 (15.86 - 115.49)	135.74 (62.28 - 196.93)	130.88 (51.06 - 270.97)
IL-17	medium	10.00 (10.00 - 19.15)	10.00 (10.00 - 11.35)	10.00 (10.00 - 13.63)	10.78 (10.00 - 41.59)
	AWA	10.00 (10.00 - 31.93)	25.59 (10.00 - 60.03)	18.09 (10.00 - 41.01)	27.41 (12.39 - 82.30)
	SEA	11.52 (10.00 - 38.83)	19.11 (10.00 - 44.83)	10.00 (10.00 - 37.55)	38.63 (11.51 - 64.09)
	BCG	39.70 (10.00 - 74.94)	36.89 (20.00 - 174.11)	65.54 (20.62 - 118.98)	109.85 (22.72 - 155.26)
TNF	medium	46.29 (14.33 - 125.45)	42.53 (15.66 - 178.20)	98.50 (33.23 - 233.55)	101.56 (25.05 - 256.81)
	AWA	154.54 (85.24 - 231.39)	330.86 (167.01 - 529.55)	224.74 (101.72 - 335.88)	306.40 (122.04 - 582.24)
	SEA	119.93 (30.84 - 265.32)	201.29 (44.71 - 480.41)	94.31 (42.38 - 261.80)	172.38 (66.79 - 366.74)
	BCG	537.67 (183.00 - 1268.92)	1708.70 (738.50 - 5445.60)	1024.85 (422.46 - 2013.62)	1641.89 (675.63 - 4619.88)