Functional Studies of T Regulatory Lymphocytes in Human Schistosomiasis in Western Kenya

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Abstract. Immunoregulation is considered a common feature of Schistosoma mansoni infections, and elevated levels of T regulatory (Treg) lymphocytes have been reported during chronic human schistosomiasis. We now report that the removal of Treg (CD4+/CD255/CD127low lymphocytes) from peripheral blood mononuclear cells (PBMCs) of S. mansoni–infected individuals leads to increased levels of phytohemagglutinin (PHA)-stimulated interferon gamma (IFNγ) production and decreased interleukin-10 (IL-10) responses. Exposure to schistosome antigens did not result in measurable IFNγ by either PBMC or Treg-depleted populations. Interleukin-10 responses to soluble egg antigens (SEA) by PBMC were unchanged by Treg depletion, but the depletion of Treg greatly decreased IL-10 production to soluble worm antigenic preparation (SWAP). Proliferative responses to PHA increased upon Treg removal, but responses to SEA or SWAP did not, unless only initially low responders were evaluated. Addition of anti-IL-10 increased PBMC proliferative responses to either SEA or SWAP, but did not alter responses by Treg-depleted cells. Blockade by anti-transforming growth factor-beta (TGF-β) increased SEA but not SWAP proliferative responses by PBMC, whereas anti-TGF-β increased both SEA- and SWAP-stimulated responses by Treg-depleted cultures. Addition of both anti-IL-10 and anti-TGF-β to PBMC or Treg-depleted populations increased proliferation of both populations to either SEA or SWAP. These studies demonstrate that Treg appear to produce much of the antigen-stimulated IL-10, but other cell types or subsets of Treg may produce much of the TGF-β. The elevated levels of Treg seen in chronic schistosomiasis appear functional and involve IL-10 and TGF-β in antigen-specific immunoregulation perhaps leading to regulation of immunopathology and/or possibly decreased immunoprotective responses.

INTRODUCTION

Schistosomiasis is a common neglected tropical disease that affects more than 230 million people worldwide.1 Multiple studies have described major roles for immune responses with regard to both morbidity and resistance to reinfection in human schistosomiasis.2–7 T Regulatory (Treg) lymphocytes are unique subpopulations of T cells involved in immune homeostasis and tolerance8–11 and their elevation has been reported in human schistosomiasis.2,4,12,13 Regulation of effector T cells during chronic antigenic exposure, such as in schistosomiasis, may protect the host from excessive pathology, but may also impair effective immune-mediated resistance to reinfection. However, Treg quantification and functionality during disease states remains controversial, in part because of the state of flux of reliable markers and the challenge of antigen-specific and nonspecific functional assays.14,15 In this study we have further characterized Treg from individuals with schistosomiasis and evaluated the functional capabilities of their Treg in regard to schistosome antigen-specific and mitogen-stimulated proliferative and cytokine responses.

METHODOLOGY

Study area and study population. This study was done in Kisumu County in western Kenya. Kisumu is located on the shores of Lake Victoria, where transmission of S. mansoni is well documented.13,16–18 The study participants (median age 31 years, range 18–63 years) were men employed as sand harvesters or car washers, occupations that expose them daily to schistosome transmission in Lake Victoria.16,18

Ethical considerations. The objective of the study was explained to all study participants and written consent was obtained from each subject. The research protocol was approved by the Scientific Steering Committee of the Kenya Medical Research Institute (SSC-KEMRI), KEMRI/Scientific Ethical Review Unit (Protocol No. 1913) and the Institutional Review Boards at the University of Georgia (Protocol No. 00004080) and the Centers for Disease Control and Prevention. Centers for Disease Control and Prevention investigators were determined to be nonengaged from a human subject perspective.

Fecal examinations for helminth parasites. Infection by S. mansoni, Ascaris lumbricoides, Trichuris trichiura, and hookworm was determined by Kato–Katz fecal examination based on three consecutive stools, two slides each.19 The intensity of the infection was obtained for S. mansoni as eggs per gram of feces (EPG) and the presence or absence of eggs of the three soil-transmitted helminths (STH) was recorded. The intensity of S. mansoni infection was classified according to the World Health Organization (WHO 2013) guidelines20 as light (1–99 EPG), moderate (100–399 EPG), and heavy infections (≥400 EPG). Individuals positive for S. mansoni were treated with 40 mg/kg Praziquantel (PZQ) and anyone positive for STH was treated with 400 mg albendazole.

Peripheral blood mononuclear cell (PBMC) immunophenotyping, isolation, and Treg depletion. Heparinized blood (8–20 mL) was collected by sterile venipuncture after diagnosis by Kato–Katz assay and before treatment with PZQ, and immunophenotyping was performed using the following antibodies: anti-CD3, anti-CD4, anti-CD25, anti-CD127, and anti-FOXp3 directly labeled with FITC, PE, PerCP, APC or AF 488. Anti-CD3 (AF 488, PerCP or PE conjugated) clone,
Peripheral blood mononuclear cells were obtained by density gradient centrifugation of heparinised venous blood over Fico/Lite (Atlanta Biologics, Atlanta, GA) within 2 hours of collection. Peripheral blood mononuclear cell were split into two aliquots consisting of ≥ 5.0 x 10^6 cells each and either not processed further or processed for depletion of CD25γδ cells using anti-CD25 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, CD25 + cells were magnetically labeled with 40 μL of CD25 MicroBeads II. The cell suspension was then loaded onto a MACS® Column (Miltenyi Biotec), which was placed in the magnetic field of a MACS Separator. The flow-through containing unlabeled cells was collected (CD25 Treg-depleted population). Following the Treg depletion process, aliquots of both unprocessed PBMC and Treg-depleted populations were analyzed for expression of CD3, CD4, CD25, and CD127 as described above. Flow cytometry was performed using the Four-color FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software version 10.1 (TreeStar, Ashland, OR). Gating was done using the fluorescence minus one procedure as reported previously in detail.13

To measure lymphocyte proliferation, cells (2 x 10⁶) were either cultured in the presence of PHA and media control or S. mansoni antigens (SEA and SWAP) and media control, for 3 and 5 days respectively, as detailed previously for cytokine production except cultures were 200 μL in volume in flat-bottom 96 well plates (CoStar, Corning, NY). Proliferation was measured by quantifying DNA levels at the time of harvest using BrdU kits per the manufacturer instructions (Roche Diagnostics, Mannheim, Germany). Therefore, for the last 16 hours of incubation, 20 μL/well of BrdU labeling solution was added to each well. On harvesting, the labeling medium was removed by centrifuging the culture plate at 300 g for 10 minutes followed by flicking the media off the plate. Cells were fixed and DNA denatured by the addition of 200 μL/well FixDenat that was provided with the kit. This was followed by the incubation of the plate for 30 minutes at RT. The FixDenat solution was then removed by flicking and tapping. Anti-BrdU-POD (100 μL/well 1:100 dilution) was added to each well and incubated for 90 minutes at RT. Anti-BrdU was removed and the wells rinsed three times with 300 μL/well of wash buffer. Subsequently, 100 μL/well of substrate was added and the plate incubated for 5 minutes at RT. The reaction was stopped by the addition of 25 μL/well of 1 M H₂SO₄ and absorbance was read at 450 nm on a Spectramax Emax plate reader (Molecular Devices).

Potential impact of anti-IL-10 and/or anti-TGF-β on antigen-stimulated proliferation. To evaluate the effect of blockade of IL-10, TGF-β or both IL-10 and TGF-β on proliferation assay cultures exposed to either SEA or SWAP, cultures of PBMC or Treg-depleted populations were incubated in parallel with 20 μL (100 μg/mL) of anti-IL-10 monoclonal antibody (mAb), clone: JES3-9D7; 20 μL (100 μg/mL) antihuman TGF-β mAb clone: 19D8; or irrelevant isotype-control mAbs (purified rat IgG1 clone: RTK2071; purified mouse IgG1, clone: MOPC-21) for each of the anti-cytokine mAbs. All mAbs were from BioLegend and were added to make a final concentration of 10 μg/mL in culture.
Statistical analysis. Data were entered into Microsoft Access 2010 databases. Individual datasets were generated using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk, NY). GraphPad Prism version 6 for windows (GraphPad Software, San Diego, CA) was used for statistical analyses and for preparing graphs. Correlations between lymphocyte populations were examined using Spearman’s correlation test. Differences in cytokine production and proliferative responses between total and depleted lymphocytes were evaluated using the Wilcoxon matched-pairs signed rank test. Tests were considered statistically significant at \( P < 0.05 \).

RESULTS

Epidemiological and demographic characteristics of the study participants. The study participants were at high risk of acquiring schistosomiasis due to occupational exposure as either sand harvesters or car washers who work in shallow water along the shores of Lake Victoria. Essentially, all sand harvesters (\( N = 33 \)) are lifelong residents of a village endemic for \( S. mansoni \) and have been persistently exposed since they were infants. More than 85% of the car washers (\( N = 45 \)) were initially exposed to possible transmission of \( S. mansoni \) as adults, when they began their employment as car washers.\(^{16} \) At the time of blood sample collection for these studies, the arithmetic mean intensity of \( S. mansoni \) infection for sand harvesters was 374 EPG (range 8–2,309) and for car washers was 91 EPG (range 4–1,325) (Table 1). None of the participants were coinfected with any of the three soil-transmitted helminths.

Phenotyping for Treg lymphocyte markers in whole blood by four-color flow cytometry. Comparing whole blood from subjects with schistosomiasis by direct immunofluorescent staining for expression of CD3, CD4, FoxP3, CD25hi, and CD127, we compared different cell surface marker combinations that have been used to define Treg cells.\(^{9,23,24} \) We found that the percentages of CD4+/FoxP3+ lymphocytes did not differ from those of CD4+/CD25hi lymphocytes (\( P = 0.339 \)). Furthermore, linear regression analysis showed that the percentages of CD4+/FoxP3+ and CD4+/CD25hi/CD127low lymphocytes in a given person’s peripheral blood correlated (\( r = 0.69; \ P = 0.0007 \)), and the percentages of CD4+/CD25hi cells and CD4+/CD25hi/CD127low cells were highly correlated (\( r = 0.90; \ P < 0.0001 \)) (Figure 1). Based on these findings and access to four-color flow cytometry, we have considered that our CD3+/CD4+/CD25hi and CD3+/CD4+/CD25hi/CD127low populations represent a reasonable consensus standard set of markers for Treg.\(^{15,24} \)

Assessment of the effectiveness of Treg lymphocyte depletion by single-step separation on anti-CD25 magnetic beads. We evaluated the ability to selectively deplete Treg cells (CD3+/CD4+/CD25hi cells and CD3+/CD4+/CD25hi/CD127low cells) from PBMC preparations with anti-CD25 immunomagnetic bead separation. Flow cytometric analysis of cells before and after separation demonstrated effective removal of CD4+/CD25hi cells from almost all PBMC preparations (Figure 2A) and highly effective removal of CD4+/CD25hi/CD127low cells (Figure 2B) from all PBMC preparations.

Production of IFNγ and IL-10 by PBMCs and following depletion of Treg lymphocytes. Evaluation of the ability of an individual’s PBMC and Treg-depleted PBMC to produce IFNγ or IL-10 was determined following PHA (3 days) or SEA or SWAP (5 days) stimulation of in vitro cultures. On the

| Characteristic                     | Car washers (\( N = 45 \)) | Sand harvesters (\( N = 33 \)) |
|-----------------------------------|-----------------------------|---------------------------------|
| Age in years, median (range)      | 27 (18–63)                  | 32 (23–53)                      |
| Years worked, median (range)      | 8 (1–40)                    | 10 (3–30)                       |
| Height in centimeters, median (range) | 174 (161–187)              | 174 (157–185)                   |
| Weight in kilograms, median (range)     | 63.6 (46–85.8)        | 62 (44.4–84.4)                  |
| \( S. mansoni \) infection intensity | Light (1–99 EPG), n (%) | 33 (73%)                        |
|                                   | Moderate (100–399 EPG), n (%) | 7 (16%)                         |
|                                   | Heavy (≥400 EPG), n (%)     | 5 (11%)                         |
appropriate day, culture supernatant fluids were collected, stored at −20°C and later assayed together by cytokine-specific ELISA assays against standard curves. Peripheral blood mononuclear cells from most participants made negligible levels of IFNγ in response to PHA (Figure 3A), but most did produce IL-10 (Figure 3B). However, upon removal of Treg the reverse was true. Treg-depleted PBMC produced significantly more IFNγ (P = 0.0001) and significantly less IL-10 (P = 0.0012) compared with unseparated cells in response to PHA. The SEA- or SWAP-stimulated IFNγ responses of PBMC were essentially nil and did not change upon removal of Treg (data not shown). Interleukin-10 production in response to SEA was mixed, but responses of most individuals declined following Treg removal (Figure 4A). Soluble worm antigenic preparation stimulation of cultures of Treg-depleted cells demonstrated significantly decreased levels of IL-10 compared with those produced by parallel PBMC cultures (P = 0.0078) (Figure 4B), similar to what was seen with PHA stimulation.

Treg-depletion increases proliferation in response to PHA but not to SEA or SWAP. Using the BrdU proliferation assay,25 we confirmed that optimal responses were obtained at day 3 when PBMC cultures were exposed to the mitogen PHA and by 5 days when cultured with SEA or SWAP. Cultures from those optimum response days are presented in Figure 5. We then compared responsiveness of PBMC and Treg-depleted populations from the same individual to evaluate the effect of Treg removal on PHA-, SEA-, or SWAP-stimulated proliferation. Paired analyses demonstrated that proliferative responses to PHA significantly increased in the Treg-depleted cultures compared with the total PBMC (P = 0.02) (Figure 6A). By contrast, Treg depletion did not significantly increase proliferative responses to SEA (P = 0.3088) or to SWAP (P = 0.126) (Figure 6B and C). When we focused on those participants with very low PBMC responses to antigen (an arbitrary cutoff for very low responses was used; O.D. ≤ 0.150), we observed that Treg removal did result in significantly increased responsiveness to SWAP (P = 0.0013, Figure 6D) and marginally increased responses to SEA (P = 0.0528, Figure 6E).

Addition of anti-IL-10 increases PBMC responses, but not Treg-depleted responses to SEA and SWAP. Addition of monoclonal anti-IL-10, but not a monoclonal isotype control, to parallel cultures of individuals’ PBMC and Treg-depleted cell populations led to increased proliferative responses to both SEA and SWAP by PBMC (P < 0.0001 for both), but Treg-depleted populations did not exhibit consistently increased responsiveness (P = 0.4437 and P = 0.7740, respectively) (Figure 7A–D).

Addition of anti-TGF-β increases Treg-depleted cell responses to both SEA and SWAP, but only PBMC responses to SEA. In contrast to anti-IL-10, addition of
monoclonal anti-TGF-β, but not a monoclonal isotype control, to parallel cultures of individuals' PBMC and Treg-depleted cell populations resulted in increased proliferation to SEA by both a person's PBMC \( (P < 0.0001) \) and their parallel cultures of Treg-depleted cells \( (P = 0.0244) \) (Figure 8A and B). Addition of anti-TGF-β to parallel cultures exposed to SWAP yielded a different pattern of change due to Treg-depletion. Anti-TGF-β failed to consistently alter PBMC responses to SWAP \( (P = 0.9408) \), but did augment SWAP-stimulated responses by parallel cultures of Treg-depleted cells \( (P = 0.023) \) (Figure 8C and D).

Co-addition of anti-IL-10 and anti-TGF-β to cultures of PBMC or Treg-depleted cells yields augmented proliferative responses by both types of cultures to both SEA and SWAP. Addition of both anti-IL-10 and anti-TGF-β, but not parallel monoclonal isotype controls, to cultures of individuals' PBMC and Treg-depleted cells led to increased responses by both types of cultures upon exposure to either SEA or SWAP. This is shown in Figure 9A–D, where PBMC responses to SEA \( (P = 0.0110) \) and SWAP \( (P = 0.0296) \) increased as did the proliferative responses of Treg-depleted cultures \( (P = 0.0005 \text{ and } P = 0.0159, \text{ respectively}) \).

DISCUSSION

Schistosomiasis remains a major public health problem in Kenya and many parts of sub-Saharan Africa. National Neglected Tropical Disease programs in most of those countries impacted are making strides to control morbidity. However, reinfection rates after annual mass drug administration (MDA) with PZQ can result in the continued high prevalence of infection. Nevertheless, at least partial resistance to reinfection may occur in some individuals after multiple rounds of treatment and reinfection. Understanding human immune responses against schistosomal antigens and their regulation has been the topic of multiple groups and studies, and elevated levels of Treg has been reported by several studies. The functional activities of such Treg in human schistosomiasis have been discussed but remain only partly understood. A continued understanding and characterization of these regulatory cells and their impact on schistosome antigen-specific responses is therefore important for our understanding of this complex infection of millions of people that characteristically presents as a chronic, systemic antigenic exposure.

The current study continues this line of research in regard to Treg-mediated immunoregulation of mitogen-stimulated and schistosomal antigen-specific cytokine production and lymphocyte proliferation in an occupational setting of repeated infections and reinfections. Evidence of possible Treg-mediated Th1 regulation by those harboring schistosomes is seen upon PHA exposure of cultures of PBMC versus Treg-depleted populations, where PBMC from almost all infected individuals fail to produce IFNγ unless Treg

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are removed (Figure 3A). By contrast, their PBMC responded to PHA by production of IL-10, but this ability was greatly reduced on depletion of Treg, indicating that much of the stimulated IL-10 is likely being produced by Treg (Figure 3B). Neither SEA nor SWAP stimulated IFN-γ production by either cell populations, but these antigenic preparations did induce IL-10 production by PBMC cultures, and this ability was decreased in Treg-depleted cultures from most individuals (Figure 4A and B), albeit not significantly in regard to SEA, suggesting that during human schistosomiasis Treg cells are a main source of immunoregulatory IL-10. Interleukin-10 has previously been correlated with control of pathogenesis, reduction of morbidity and prolonged survival in human schistosomiasis, but has also been linked to a decreased resistance to reinfection.

Peripheral blood mononuclear cell proliferation patterns from these participants in response to PHA, SEA, or SWAP are strikingly similar to those reported previously where responses to PHA and SWAP are often high, whereas those to SEA are modest or low. Mean PBMC proliferative responses to PHA were augmented by depletion of Treg (Figure 5A), but the overall mean responses to SEA and SWAP were not and expressed considerable heterogeneity (Figure 5B and C). Low responders (selected arbitrarily to have O.D. values ≤ 0.150) to SWAP did increase significantly upon Treg depletion (Figure 5D). However, although most SEA low responders increased with the removal of Treg, the mean of the Treg-depleted responses did not achieve statistical significance (Figure 5E). These results indicate that Treg immunoregulatory activity is more readily apparent in PBMC cultures that respond very poorly to these antigens.

The addition of neutralizing anti-IL-10 to antigen-driven PBMC or whole blood cultures from human schistosome patients has been studied extensively and has usually led to increased proliferation or cytokine responses. We also observe that anti-IL-10 mAb is able to alleviate IL-10-mediated immunoregulation of SEA-stimulated proliferation (Figure 7A) and we have extended these observations to show that anti-IL-10 does not change the SEA-stimulated response of Treg-depleted cultures (Figure 7B). The same is true for SWAP-stimulated PBMC or Treg-depleted cultures cocultured with anti-IL-10 (Figure 7C and D). In conjunction with the data in Figure 3B, which showed that depletion of Treg led to a loss of production of IL-10 upon PHA exposure, this finding provides evidence that a major mode of Treg immunoregulation is mediated through the production of IL-10.

The ability of anti-TGF-β mAb to alter the SEA- or SWAP-stimulation of PBMC or Treg-depleted cultures presents a different picture than that with anti-IL-10. Anti-TGF-β again augments the PBMC response to SEA, but also augments the
Treg-depleted response to SEA (Figure 8A and B), likely indicating that non-Treg cells are producing much of the immunoregulatory TGF-β. This appears to also be true for SWAP-stimulated Treg cultures (Figure 8D), however the failure of anti-TGF-β to increase SWAP-stimulated proliferation may indicate that the immunoregulatory effect of SWAP-stimulated IL-10 in these cultures is too strong to overcome by blocking with anti-TGF-β. On Treg removal, and with it the source of most of the IL-10 production, anti-TGF-β can effectively block the regulatory TGF-β from non-Treg cells, resulting in responsiveness.

When anti-IL-10 mAb and anti-TGF-β mAb are both added to SEA-stimulated PBMC or Treg-depleted cultures the mean proliferative responses of both types of cultures are significantly increased (Figure 9A and B). The same is true when this combinatorial blockade is included in SWAP-stimulated PBMC or Treg-depleted cultures (Figure 9C and D). It is apparent that blockade of both immunoregulatory cytokines, IL-10 and TGF-β, is sufficient to allow augmented antigen-specific responses to both SEA and SWAP by cells from most infected individuals, and in some cases the increases are quite striking.
substantial. Other studies have also reported that Treg function through the production of anti-inflammatory cytokines, for example, IL-10 and/or TGF-β in a variety of immune-mediated conditions.\textsuperscript{44–47} Immunoregulation is an integral part of chronic human schistosomiasis.\textsuperscript{34} Elevated Treg and the involvement of IL-10\textsuperscript{4,12} and even TGF-β\textsuperscript{48–50} have been reported in studies of people with schistosomiasis or other chronic helminthic infections. However, the antigen-specific functional abilities of the elevated Treg populations in persons with schistosomiasis have not been generally reported. Here we provide evidence that Treg, IL-10, and TGF-β are involved in the regulation of SEA and SWAP responses. It appears that Treg produce much of the IL-10 stimulated by either of the antigens, although the source of at least some of the TGF-β remains unclear. Other possible sources of antigen-stimulated TGF-β have been reported and

**Figure 7.** (A and B) Peripheral blood mononuclear cells and their parallel T regulatory (Treg)-depleted populations were cultured in the presence of schistosome soluble egg antigen (SEA) plus isotype control monoclonal antibody (mAb) (IsoControl) compared with culture in the presence of SEA plus mAb against interleukin-10 (anti-IL-10). (C and D) Peripheral blood mononuclear cell and their parallel Treg-depleted populations were cultured in the presence of schistosome soluble worm antigenic preparation (SWAP) plus IsoControl compared with culture in the presence of SWAP plus anti-IL-10. The levels of proliferation in the cultures were determined by incorporation of BrdU/ labeled anti-BrdU expressed as optical density values (O.D.) as Experimental (E) or Control (C). E–C O.D. values for each pair are plotted and were analyzed by Wilcoxon matched-pairs signed rank test. \( P \) values for differences in the mean E–C values are given above each pair in each panel.
include epithelial cells, fibroblasts, and immune-associated cells such as macrophages and eosinophils. Regulatory B cells from schistosome patients also produce IL-10 as can CD8+ cells IL-10 and should perhaps be further investigated in schistosomiasis. Regulation of responses to SEA may be responsible for the control of morbidity caused by granulomatous reactions to those schistosome eggs that fail to be excreted and become lodged in the tissues.
chronic infection induced–immunoregulatory mechanisms demonstrated here, Treg, IL-10 and TGF-β may, therefore, curtail severe disease in the majority of individuals infected with *S. mansoni* and other helminthic infections.\(^6,34,54\) It is also possible that these same immunoregulatory mechanisms, when generated in response to SWAP may participate in regulation of otherwise protective immune responses against multiple reinfections.\(^36,55\)
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