

# T-Helper 17 Cells Are Associated With Pathology in Human Schistosomiasis

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**Background.** Schistosome infections are often clinically silent, but some individuals develop severe pathological reactions. In several disease processes, T-helper 17 (Th17) cells have been linked to tissue injuries, while regulatory T cells (Tregs) are thought to downmodulate inflammatory reactions. We assessed whether bladder pathology in human *Schistosoma haematobium* infection is related to the balance of Th17 cells and Tregs. We used a murine model of *Schistosoma mansoni* infection to further investigate whether the peripheral profiles reflected ongoing events in tissues.

**Methods.** We characterized T-helper cell subsets in the peripheral blood of children residing in a *S. haematobium*-endemic area and in the peripheral blood, spleen, and hepatic granulomas of *S. mansoni*-infected high-pathology CBA mice and low-pathology C57BL/6 mice.

**Results.** *S. haematobium*-infected children with bladder pathology had a significantly higher percentage of Th17 cells than those without pathology. Moreover, the Th17/Treg ratios were significantly higher in infected children with pathology, compared with infected children without pathology. Percentages of interleukin 17-producing cells were significantly higher in spleen and granulomas of CBA mice, compared with C57BL/6 mice. This difference was also reflected in the peripheral blood.

**Conclusions.** This is the first study to indicate that Th17 cells may be involved in the pathogenesis of human schistosomiasis.

**Keywords.** Schistosomiasis; pathology; Th17 cells.

More than 200 million people are infected with schistosomes worldwide [1], with the majority of infections occurring in Africa [2]. Although infections are often clinically silent, severe morbidity occurs in a subset of the population. The main pathology is induced by a granulomatous tissue reaction against the parasite eggs [3–5].

The mechanisms underlying the development of pathology are not well-defined. Schistosome morbidity has

been shown to vary directly with the egg count [3, 6], but immunologic differences have also been associated with pathology outcomes [7, 8]. Understanding the molecular mechanisms involved in schistosome immunopathogenesis may have important implications for disease intervention strategies not only for schistosomiasis [4, 9] but also for other parasitic diseases.

Studies in interleukin 13- and interleukin 4 (IL-4)-deficient schistosome infected-mice have demonstrated that T-helper 2 (Th2) cell cytokines are involved in the granulomatous response and fibrosis [10]. However, the notion that Th2 cell responses may downmodulate inflammation and participate in tissue repair [11, 12] prompts the question of which other cell subset(s) might be involved in the development of severe immunopathology.

Recent studies in murine schistosomiasis have revealed a critical pathogenic role for interleukin 17

Received 12 May 2012; accepted 31 July 2012; electronically published 19 October 2012.

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**The Journal of Infectious Diseases** 2013;207:186–95

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DOI: 10.1093/infdis/jis654

(IL-17)-producing CD4<sup>+</sup> cells (T-helper 17 [Th17] cells) [9, 13]. Regarding regulatory T cells (Tregs), it is known that helminths can induce Treg responses associated with parasite survival [14, 15], whereas during schistosomiasis Tregs seem to control pathology [16, 17]. Studies on the differentiation of Th17 cells and Tregs have shown a possible reciprocal link between these T cell subsets, but the recent findings in murine models suggesting that Th17 cells exacerbate and Tregs curtail the development of pathology [4] have seldom been extrapolated to humans. Here, we investigated the role of Th17 cells in *Schistosoma haematobium*-infected subjects with and without pathology to determine whether an imbalance between Th17 cells and Tregs is associated with pathology in human schistosomiasis. We therefore assessed the expression of the key T-helper cell transcription factors FOXP3, ROR $\gamma$ t, T-bet, and GATA3, as well as the CD4<sup>+</sup> T cell-related cytokines interleukin 10 (IL-10), IL-17, interleukin 22 (IL-22), interferon  $\gamma$  (IFN- $\gamma$ ), and IL-4. In humans, the only readily available immune compartment is the peripheral blood, and it can be argued that the findings might not reflect the immune activity in target organs. Given that infection with *S. haematobium* is an extremely difficult model to create and is not readily available [18], we used the well-characterized murine model of *Schistosoma mansoni* infection in high (severe)-pathology CBA mice and low (mild)-pathology BL/6 mice and investigated whether the phenotype of CD4<sup>+</sup> T cells in the periphery reflects the profile seen in target tissues.

## MATERIALS AND METHODS

### Study Population

Our study took place in Pakh, a town in northern Senegal, where *S. mansoni* and *S. haematobium* are endemic. A total of 213 subjects aged 5–55 years participated in the study. Of these, 87 subjects did not have complete parasitological data. Ultrasonography was performed on 200 of the 213 participants. Complete data (parasitology and ultrasonography findings) were available for 126 subjects. Our aim was to study *Schistosoma*-associated morbidity caused by a single *Schistosoma* species. Because *S. haematobium* was more prevalent than *S. mansoni* and because children displayed higher prevalences of infection and morbidity due to *S. haematobium*, we focused on *S. haematobium*-infected children. On this basis, we defined 3 groups of children aged 5–14 years: children negative for both *S. haematobium* and *S. mansoni*, with no schistosome-related pathology (S–P–; n = 10), *S. haematobium*-infected children with no schistosome-related pathology (S+P–; n = 6), and *S. haematobium*-infected children with *S. haematobium*-related pathology (S+P+; n = 10). This study was approved by the review boards of the Institute of Tropical Medicine of Antwerp (permit B30020095424) and Le Comité National d’Ethique de la Recherche en Santé of Senegal (permit

0044MSPHP/DS/CNERS). For all participants, written informed consent was obtained from a parent or legal guardian.

### Parasitology and Ultrasonography

Participants were assessed for both *S. mansoni* and *S. haematobium* infections. Fecal *S. mansoni* egg counts were determined on duplicate Kato-Katz slides (2 × 25 mg) that contained samples obtained on 2 different days. The number of *Schistosoma haematobium* eggs in 10 mL of urine collected on 2 different days was assessed using filters with 12- $\mu$ m pores.

Ultrasonography was used according to the Niamey guidelines [19] to detect textural abnormalities in bladder tissue indicative of pathologic lesions associated with *S. haematobium*, and findings were translated into scores by an experienced ultrasonographer. For *S. mansoni*-specific morbidity, liver image patterns were categorized as *S. mansoni*-specific hepatic morbidity. All examinations were performed by the same clinician, who was blinded to the participant’s infection status.

A thick smear was performed to test for malaria infection. All participants were screened by a physician to exclude individuals with other pathologies unrelated to schistosomes, and a full blood count was performed using the AcT 5 Diff hematology analyzer (Beckman Coulter).

### Cell Isolation and Fixation

Peripheral blood mononuclear cells (PBMCs) were isolated within 4 hours of blood collection by Ficoll density gradient centrifugation. One million cells were fixed in fixation buffer (eBioscience) for 1 hour; frozen in 10% fetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO), and Roswell Park Memorial Institute (RPMI) medium; and stored at –80°C.

### Cell Stimulation

To assess T-cell cytokines, 1 × 10<sup>6</sup> PBMCs were stimulated for 6 hours with 100 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich) and 1  $\mu$ g/mL ionomycin (Sigma-Aldrich). Brefeldin A (BFA; 10  $\mu$ g; Sigma-Aldrich) was added 2 hours later, and cells were then incubated at 37°C in 5% CO<sub>2</sub> for 4 more hours. Stimulated cells were fixed in 2% paraformaldehyde (PFA; Sigma Aldrich) for 15 minutes; frozen in 10% FCS, 10% DMSO, and RPMI medium; and stored at –80°C.

### Flow Cytometry Analysis

Fixed PBMCs-using eBioscience fixation buffer for assessment of transcription factors and PFA for and assessment of intracellular cytokines-were thawed, washed once in 10% FBS and RPMI medium, and washed once in phosphate-buffered saline (PBS; Fresenius Kabi). Cells were permeabilized at room temperature and then stained for 30 minutes at 4°C in permeabilization buffer containing anti-Fc $\gamma$ R (eBioscience), to inhibit nonspecific binding. For measurement of transcription

factor expression,  $2.5 \times 10^5$  PBMCs were stained with 2 different panels. Panel 1 contained AmCyan-labeled anti-CD3 (BD Biosciences), PE-Cy7-labeled anti-CD4 (BD Biosciences), PE-labeled anti-ROR $\gamma$ t (eBioscience), PerCP-Cy5.5-labeled anti-T-bet (eBioscience), and eFluoro660-labeled anti-GATA3 (eBioscience); panel 2 contained APC-labeled anti-CD3 (BD Biosciences), PerCP-labeled anti-CD4 (BD Biosciences), PE-Cy7-labeled anti-CD25 (BD Biosciences), and FITC-labeled anti-FOXP3 (eBioscience). For cytokines,  $2.5 \times 10^5$  PBMCs were stained with 2 panels. Panel 1 contained AmCyan-labeled anti-CD3 (BD Biosciences), PerCP-labeled anti-CD4 (BD Biosciences), AF488-labeled anti-IL17 (eBioscience), PE-labeled anti-IL-22 (R and D systems), PE-Cy7-labeled anti-IL-4 (eBioscience), and APC-labeled anti-IFN- $\gamma$  (BD Biosciences); panel 2 contained AmCyan-labeled anti-CD3 (BD Biosciences), PerCP-labeled anti-CD4 (BD Biosciences), and PE-labeled anti-IL-10 (BD Biosciences). Stained cells were washed; suspended in 0.5% BSA, 2 mM ethylenediaminetetraacetic acid, and PBS; and acquired in a FACSCanto instrument (BD Biosciences). Results were analyzed by FlowJo software, version 8.8.6 (Tree Star).

### Murine Infection Model

Female CBA/J and C57BL/6 mice aged 5–6 weeks, purchased from The Jackson Laboratory (Bar Harbor, ME), were maintained at the Animal Facility at Tufts University School of Medicine in accordance with the American Association for the Assessment and Accreditation of Laboratory Animal Care guidelines. Mice were infected using intraperitoneal injection of 85 *S. mansoni* cercariae (Puerto Rico strain) obtained from *S. mansoni*-infected *Biomphalaria glabrata* snails; snails were provided by Dr Fred Lewis (Biomedical Research Institute, Rockville, MD) through National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases contract N01AI-55270. Mice were sacrificed 8 weeks after infection. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH and with the permission of the American Association for the Assessment and Accreditation of Laboratory Animal Care (permit B2009-88).

### Murine Cell Preparations and Stimulation

To obtain granuloma cells, pooled livers from 2–3 mice were homogenized in a Waring blender, followed by sedimentation at  $1 \times g$ , extensive washing, and enzymatic digestion with 1 mg/mL of collagenase type H from *Clostridium histolyticum* (Sigma Chemical). Dead cells and erythrocytes were removed from the granuloma cell suspensions by centrifugation with Lympholyte-M (Cedarlane) according to the manufacturer's instructions. Spleens were removed, and single-cell suspensions were obtained by mechanical disruption of the tissue in complete RPMI 1640 medium supplemented with 10% FBS

(Aleken Biologicals), 4 mM L-glutamine, 80 U/mL penicillin, 80  $\mu$ g/mL streptomycin, 1 mM sodium pyruvate, 10 mM HEPES,  $1 \times$  nonessential amino acids (all from BioWhittaker), and 0.1% 2-mercaptoethanol. PBMCs were isolated from blood collected via retro-orbital bleed. Erythrocytes were lysed by incubating cells in Tris ammonium chloride buffer (pH 7.2; Sigma) for 15 minutes on ice. Cells were washed, counted, and resuspended in complete RPMI 1640.

Two million cells per milliliter were stimulated with 50 ng/mL PMA (Sigma) and 500 ng/mL ionomycin (Sigma) for 6 hours at 37°C in 5% CO<sub>2</sub>. A total of 10  $\mu$ g/mL BFA (Sigma) was added for the last 4 hours of culture.

### Murine Flow Cytometry Analysis

For assessment of IL-17, IFN- $\gamma$ , and IL-4 expression, cells were washed in FACS buffer (PBS with 0.1% BSA [Sigma] and 0.01% sodium azide [Sigma]) containing 10  $\mu$ g/mL BFA and were blocked for 10 minutes on ice in BFA-FACS buffer containing 0.3 mg/mL rat immunoglobulin G (IgG; Sigma). Cells were stained with APC-labeled anti-CD4 (BD Bioscience) for 25 minutes, washed once in BFA-FACS buffer, fixed for 15 minutes at room temperature in PBS containing 4% PFA and then washed once in FACS buffer, permeabilized for 15 minutes at room temperature with FACS buffer containing 0.1% saponin (Sigma) and 0.3 mg/mL rat IgG (Sigma), and stained for 35 minutes at room temperature with PE-labeled anti-IL-17 (BD Pharmingen), FITC-labeled anti-IFN- $\gamma$  (BD Pharmingen), or PE-labeled anti-IL-4 (BD Pharmingen). Cells were washed once with FACS buffer containing 0.1% saponin, washed twice with normal FACS buffer, and resuspended in 1% PFA for analysis. For FOXP3 expression, unstimulated cells were washed in FACS buffer, blocked for 10 minutes with rat IgG, and stained for CD4 expression, as described above. Cells were then fixed with eBioscience fixation buffer for 45 minutes at 4°C and washed with FACS buffer. APC-labeled anti-FOXP3 (eBioscience) was diluted in 0.1% saponin FACS buffer with rat IgG, and cells were stained for 45 minutes at room temperature. Cells were washed once with 0.1% saponin buffer, washed once with normal FACS buffer, and resuspended in FACS buffer for analysis. Cells were acquired on a FACS-Calibur flow cytometer, using CELLQUEST software, version 3.2.1 (Becton Dickinson). Results were analyzed using Summit V4.3 software (Dako).

### Statistical Analysis

Human data were analyzed with SPSS, version 17 (SPSS, Chicago, IL); differences between groups were evaluated by nonparametric Kruskal-Wallis H and Mann-Whitney *U* tests. Mouse data were analyzed with GraphPad Prism V5.03 for Windows (GraphPad, CA); 1-way analysis of variance was used to determine statistically significant differences

between groups. A *P* value of <.05 was considered statistically significant.

## RESULTS

### Analysis of the Study Population

Age, sex, and *S. haematobium* egg count did not differ significantly between groups, although we noted that the egg count tended to be higher in the S+P+ group, compared with the S+P− group. Among children with pathology, 80% had bladder irregularities, and 20% had bladder wall thickening. Table 1 shows the characteristics of the study groups.

### Subjects With Pathology Showed an Increase in Peripheral Blood Th17 Cells

Th17 cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>RORγt<sup>+</sup>, as shown in the gating strategy in Figure 1A. We found a significantly higher percentage of CD4<sup>+</sup>RORγt<sup>+</sup> T cells in the S+P+ group (4.9% [interquartile range {IQR}, 4.1%–5.7%]), compared with the S+P− group (2.3% [IQR, 1.9–3.9]; *P* = .03) and the S−P− group (2.8% [IQR, 2.5–4.0]; *P* = .003) (overall *P* = .008 (the Overall *P* values compare the three groups S−P−, S+P−, and S+P+); Figure 1B). Similarly, the percentage of CD4<sup>+</sup>IL-17<sup>+</sup> T cells (gating strategy shown in Figure 2A) was significantly higher in the S+P+ group (2.8% [IQR, 2.2–3.5]), compared with the S+P− group (1.5% [IQR, 1.2–2.3]; *P* = .009) (overall *P* = .025; Figure 2B). The difference in IL-17-producing CD4<sup>+</sup> T cells between the S+P− and S−P− groups fell short of statistical significance (*P* = .065).

We also assessed CD4<sup>+</sup>IL-22<sup>+</sup> T cells, which have been reported to take part in pathological reactions, and found a tendency toward a higher percentage of IL-22-producing T cells

in the S+P+ group (3.06% [IQR, 2.5–4.1]), compared with the S+P− group (2.4% [IQR, 1.8–2.8]; *P* = .065; Figure 2C).

With regard to Tregs, there were no significant differences in percentages of either CD4<sup>+</sup>FOXP3<sup>+</sup> cells (Figure 1C) or CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells (Figure 1D) between the 3 groups (gated as in Figure 1A). When expressed as ratios of Th17 cells and Tregs, we found a significantly higher ratio of CD4<sup>+</sup>RORγt<sup>+</sup> cells to CD4<sup>+</sup>FOXP3<sup>+</sup> cells in the S+P+ group (1.2 [IQR, 0.9–1.7]), compared with the S+P− group (0.5 [IQR, 0.3–0.8]; *P* = .017) and the S−P− group (0.5 [IQR, 0.4–0.8]; *P* = .004) (overall *P* = .007; Figure 1E). The ratio of CD4<sup>+</sup>RORγt<sup>+</sup> cells to CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells in the S+P+ group (7.7 [IQR, 2.1–13.6]) was also significantly higher than that in the S−P− group (3.5 [IQR, 2.0–6.1]; *P* = .019) but not in the S+P− group (4.19 [IQR, 2.5–6.5]; *P* = .083) (overall *P* = .044; Figure 1F). Similarly, IL-10-producing CD4<sup>+</sup> T cells did not significantly differ between groups (Figure 2D), but the ratio of CD4<sup>+</sup>IL-17<sup>+</sup> T cells to CD4<sup>+</sup>IL-10<sup>+</sup> T cells was significantly higher in the S+P+ group (1.2 [IQR, 0.9–1.7]), compared with the S+P− group (0.5 [IQR, 0.3–0.8]; *P* = .009) (overall *P* = .028; Figure 2E), indicating the relative abundance of IL-17-producing CD4<sup>+</sup> T cells, versus the abundance of IL-10-producing CD4<sup>+</sup> T cells, in patients with pathology.

### Th1 and Th2 Cell Levels Do Not Differ Between Study Subjects With and Those Without Pathology

We examined the Th1 cell subset as CD4<sup>+</sup>T-bet<sup>+</sup> or CD4<sup>+</sup>IFN-γ<sup>+</sup> T cells and the Th2 cell subset as CD4<sup>+</sup>GATA3<sup>+</sup> or CD4<sup>+</sup>IL-4<sup>+</sup> T cells; the gating strategy is shown in Figure 3A. No significant difference in either the frequencies of T-bet− or GATA3-expressing CD4<sup>+</sup> T cells (Figure 3B and 3C) or the frequencies of IL-4− and IFN-γ-producing CD4<sup>+</sup> T cells (Figure 3E and 3F) was found. Furthermore, there was no significant difference between the 3 groups with regard to the ratio of Th1 cells to Th2 cells, as defined by CD4<sup>+</sup>T-bet<sup>+</sup>/CD4<sup>+</sup>GATA3<sup>+</sup> cells (Figure 3D) or CD4<sup>+</sup>IFN-γ<sup>+</sup>/CD4<sup>+</sup>IL-4<sup>+</sup> T cells (Figure 3G).

### Peripheral Blood Granulocyte Levels Are Increased in Patients With Pathology

The full blood count revealed a significantly higher total number of granulocytes in the S+P+ group (5.48 × 10<sup>3</sup> granulocytes/μL [IQR, 4.4–9.5]), compared with the S+P− group (3.5 × 10<sup>3</sup> granulocytes/μL [IQR, 1.6–4.6]; *P* = .049) and the S−P− group (4.2 × 10<sup>3</sup> granulocytes/μL [IQR, 2.8–5.0]; *P* = .039) (overall *P* = .044; Figure 4A). Patients with pathology tended to have a higher level of neutrophils (3.0 × 10<sup>3</sup> neutrophils/μL [IQR, 2.0–4.5]), compared with patients without pathology (1.99 × 10<sup>3</sup> neutrophils/μL [IQR, 1.1–3.6]; Figure 4B), and their eosinophil levels were significantly increased (1.84 × 10<sup>3</sup> eosinophils/μL [IQR, 0.9–6.7] in the S+P+ group

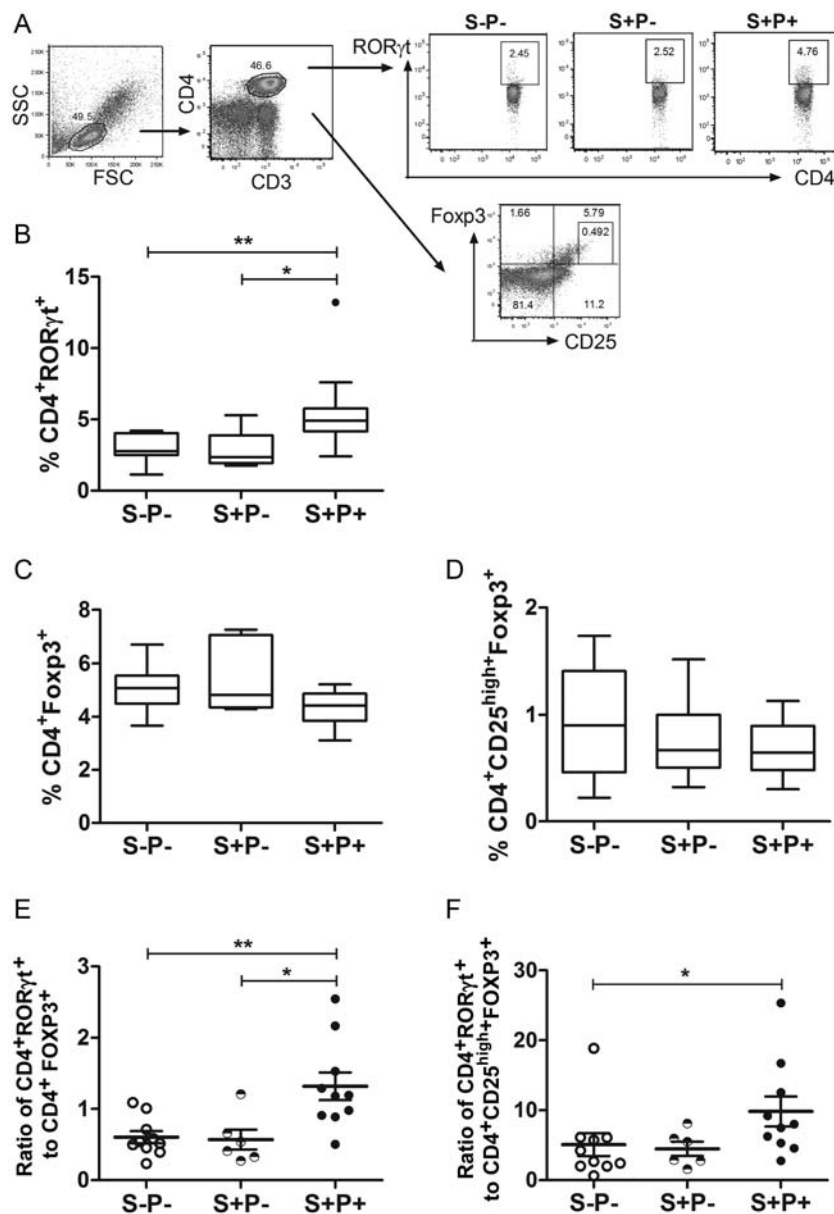
**Table 1. Characteristics of Study Subjects Aged 5–14 Years, by *Schistosoma haematobium* Infection (S) and Pathology (P) Status**

Characteristic	S−P− (n = 10)	S+P− (n = 6)	S+P+ (n = 10)	<i>P</i> <sup>a</sup>
Age, y, median (range)	10 (5–14)	8 (7–14)	9 (5–14)	.913
Males sex, no. (%)	4 (40)	2 (33.3)	7 (70)	.166
<i>S. mansoni</i> eggs per 25 mg stool	0	0	0	
<i>S. haematobium</i> eggs per 10 mL urine, no., median (IQR)	0	30.5 (10.0–70.1)	81.2 (29.2–197.7)	.159
Liver morbidity	No	No	No	
Urinary bladder morbidity	No	No	Yes	

Assessment of *S. haematobium* pathology was performed using ultrasonography. Image patterns revealing irregularities in the shape or thickness of the bladder wall or the presence of masses or pseudopolyps were translated into morbidity scores.

Abbreviations: IQR, interquartile; −, negative; +, positive.

<sup>a</sup> Comparison between the S+P− and S+P+ groups, by the  $\chi^2$  test (for sex) and by the nonparametric Mann–Whitney *U* test (for age and egg count).



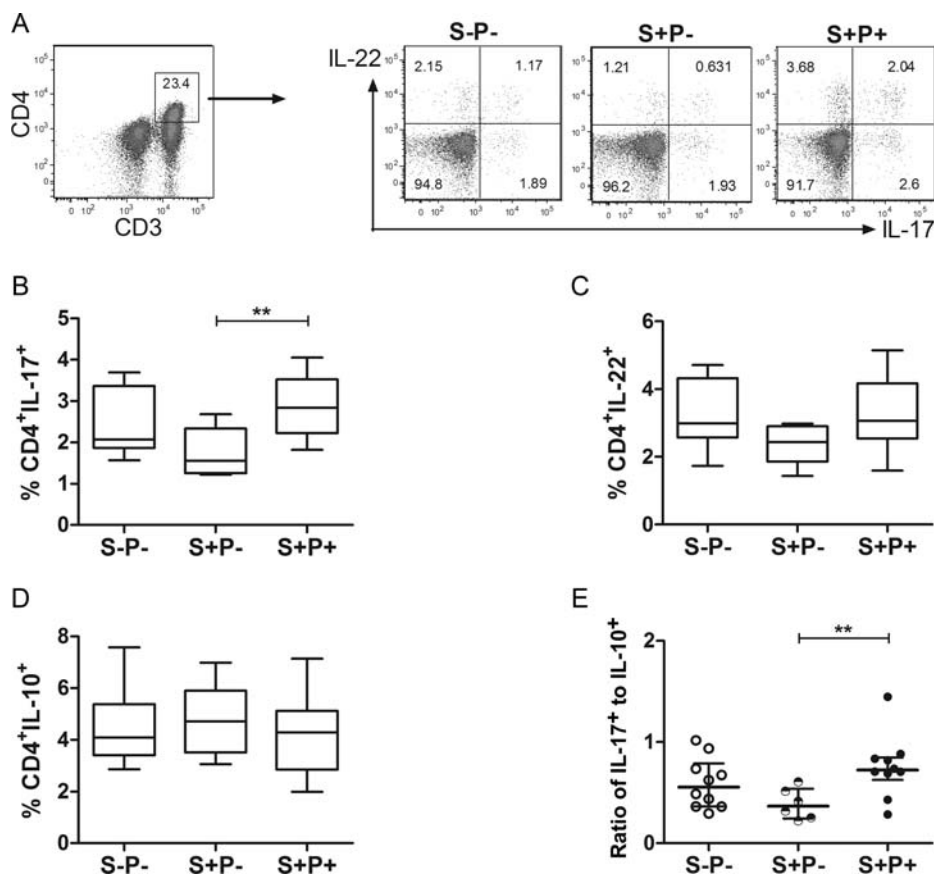
**Figure 1.** Increased ROR $\gamma$ t-expressing cells in patients with pathology. *A*, The gating strategy for determining the frequencies of ROR $\gamma$ t<sup>+</sup>, FOXP3<sup>+</sup>, and CD25<sup>high</sup>FOXP3<sup>+</sup> CD4<sup>+</sup> T cells. Total lymphocytes were gated on CD3<sup>+</sup>CD4<sup>+</sup> T cells for analysis of ROR $\gamma$ t, FOXP3, or CD25<sup>high</sup>FOXP3 expression. Box plots representing percentages of CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup> (*B*), CD4<sup>+</sup>FOXP3<sup>+</sup> (*C*), and CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> (*D*) T cells and scatter plots of the ratio of CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup> to CD4<sup>+</sup>FOXP3<sup>+</sup> (*E*) and CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup> to CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> (*F*) T cells in the S–P– (n = 10), S+P– (n = 6), and S+P+ (n = 10) groups are shown. Data are expressed as medians and interquartile ranges. *P* values are based on comparison of groups by the nonparametric Mann–Whitney *U* test. Only statistically significant *P* values are shown in the figures. \**P* < .05, \*\**P* < .01. Abbreviations: P, pathology; S, *Schistosoma haematobium* infection; –, negative; +, positive.

vs  $0.4 \times 10^3$  eosinophils/ $\mu$ L [IQR, 0.3–1.6] the S+P– group; *P* = .03; Figure 4C).

#### Th17 Cell Levels Are Increased in the Peripheral Blood and in the Spleen and Liver Granulomas of *S. mansoni*-Infected High-Pathology CBA Mice

To relate peripheral blood findings to the target tissues, we examined expression of the cytokines IL-17, IFN- $\gamma$ , and IL-4 by

PBMCs, splenocytes, and liver granuloma cells isolated from *S. mansoni*-infected CBA and BL/6 mice. In parallel with the findings involving humans, high-pathology CBA mice had a significantly higher percentage of CD4<sup>+</sup>IL-17<sup>+</sup> T cells in the peripheral blood (mean  $\pm$  SD, 0.7%  $\pm$  0.008%), compared with low-pathology BL/6 mice (mean  $\pm$  SD, 0.2%  $\pm$  0.1%; *P* < .0005; Figure 5A). The CBA mice also had significantly higher percentages of CD4<sup>+</sup>IL-17<sup>+</sup> T cells in the spleen (mean  $\pm$  SD,



**Figure 2.** Increased interleukin 17 (IL-17)–producing cells in patients with pathology. The gating strategy for determining the frequencies of interleukin 17 (IL-17)–, interleukin 22 (IL-22)–, and interleukin 10 (IL-10)–producing CD4<sup>+</sup> T cells. Total lymphocytes were gated on CD3<sup>+</sup>CD4<sup>+</sup> T cells for analysis of IL-17, IL-22, and IL-10 expression. Box plots representing median and interquartile ranges of the percentages of CD4<sup>+</sup>IL-17<sup>+</sup> (B), CD4<sup>+</sup>IL-22<sup>+</sup> (C), and CD4<sup>+</sup>IL-10<sup>+</sup> (D) T cells and scatter plots (median and IQR are shown) of the ratio of IL-17<sup>+</sup> to IL-10<sup>+</sup> cells (E) in the S–P– (n=10), S+P– (n=6), and S+P+ (n=10) groups are shown. *P* values are based on comparison of the groups by the nonparametric Mann–Whitney *U* test. Only statistically significant *P* values are shown in the figures. \*\**P* < .01. Abbreviations: P, pathology; S, *Schistosoma haematobium* infection; –, negative; +, positive.

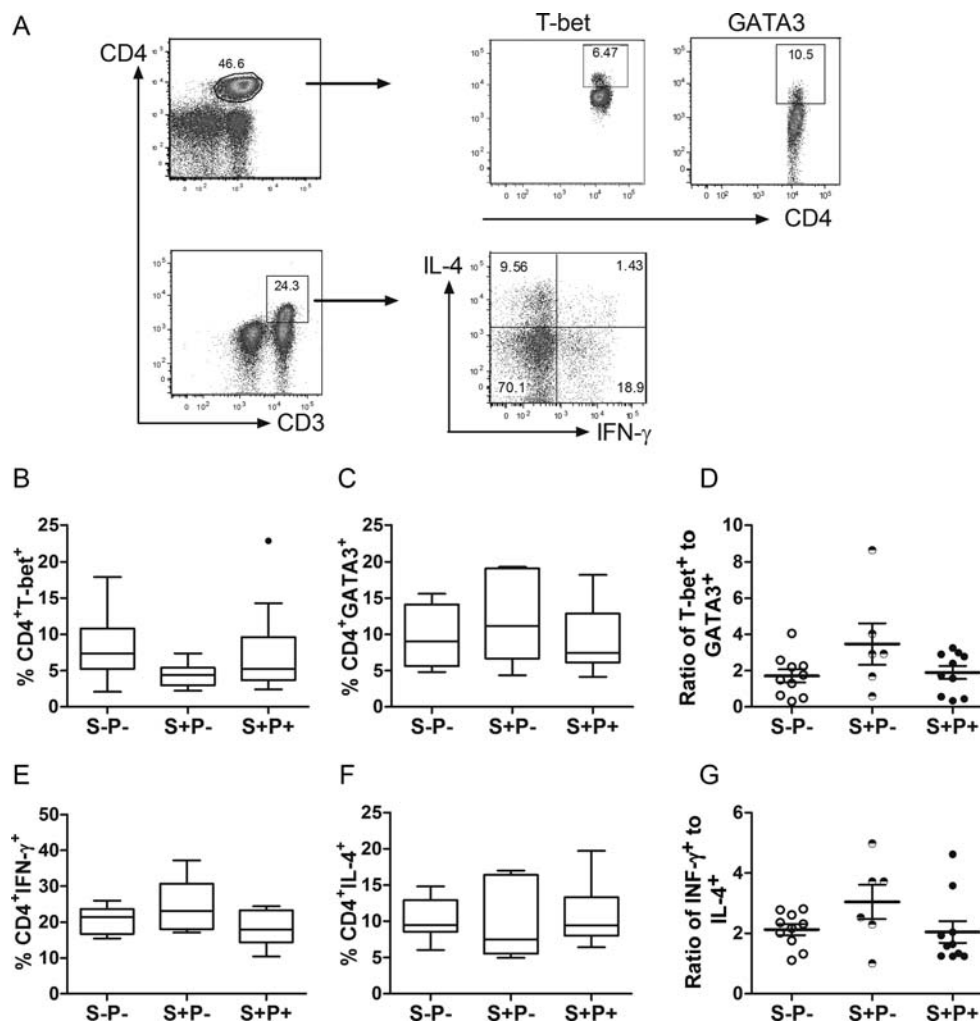
1.0% ± 0.3%; *P* < .0005) and in granulomas (mean ± SD, 8.1% ± 0.2%) (*P* = .044), compared with BL/6 mice (mean ± SD, 0.2% ± 0.05% and 1.7 ± 0.9% for spleen and liver granulomas, respectively; Figure 5B and 5C). With respect to Th1 cells, there were no statistically significant differences between the strains for IFN- $\gamma$  production in PBMCs, splenocytes, and liver granuloma cells; however, CBA mice had significantly more CD4<sup>+</sup>IL-4<sup>+</sup> T cells in PBMCs (*P* < .05) and spleen (*P* < .05; Figure 5A and 5B) but not in granuloma cells (Figure 5C).

Last, there was no significant difference between infected CBA and BL/6 mice in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in the peripheral blood (Figure 6A). Interestingly, the peripheral CD4<sup>+</sup>Foxp3<sup>+</sup> T cell levels were generally lower in infected mice than those seen in uninfected mice. Interestingly, infected BL/6 mice had a significantly higher percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in the spleen (mean ± SD, 15.3% ± 1.9%), compared with CBA mice (mean ± SD, 12.1% ± 1.2%; *P* < .005;

Figure 6B). The same trend was observed in the granulomas, although the difference did not reach statistical significance (Figure 6C). The results shown are representative of 3 separate experiments.

## DISCUSSION

The frequency of Th17 cells was significantly higher in *S. haematobium*–infected subjects with bladder pathology, compared with both infected subjects without pathology and matched uninfected individuals. However, Treg frequencies remained similar among the 3 groups. Similarly, *S. mansoni*–infected CBA mice with severe pathology showed an increased percentage of IL-17–producing CD4<sup>+</sup> T cells in the periphery, compared with low-pathology BL/6 mice. Importantly, this mirrored the increase in Th17 cells found in the liver granulomas and spleens of CBA mice. These results are in line with

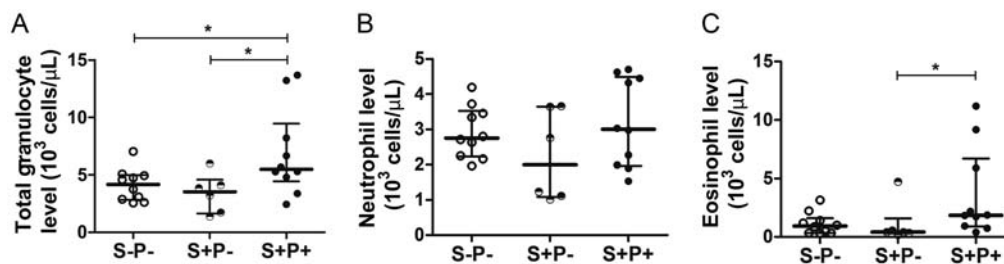


**Figure 3.** T-helper 1 cell and T-helper 2 cell levels do not differ between the three study groups. *A*, The gating strategy for determining the frequencies of T-bet or GATA3 expressing CD4<sup>+</sup> T cells and interferon  $\gamma$  (IFN- $\gamma$ )– or interleukin 4 (IL-4)–producing CD4<sup>+</sup> T cells. Total lymphocytes were gated on CD3<sup>+</sup>CD4<sup>+</sup> T cells for analysis of expression of T-bet, GATA3, IFN- $\gamma$ , or IL-4. Box plots of percentages of CD4<sup>+</sup>T-bet<sup>+</sup> (*B*) and CD4<sup>+</sup>GATA3<sup>+</sup> (*C*) T cells, scatter plots of the ratio of CD4<sup>+</sup>T-bet<sup>+</sup> to CD4<sup>+</sup>GATA3<sup>+</sup> (*D*), box plots of CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (*E*) and CD4<sup>+</sup>IL-4<sup>+</sup> (*F*) T cells, and scatter plots of the ratio of IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> to IL-4<sup>+</sup>CD4<sup>+</sup> T cells (*G*) in the S–P– (n = 10), S+P– (n = 6), and S+P+ (n = 10) groups are shown. Data are expressed as median values and interquartile ranges. *P* values were calculated using the nonparametric Mann–Whitney *U* test. No statistically significant *P* values were found. Abbreviations: P, pathology; S, *Schistosoma haematobium* infection; –, negative; +, positive.

previous findings showing that, in *S. mansoni*–infected mice, a significant increase in IL-17 production was correlated with high pathology and that neutralization of IL-17 in vivo resulted in significant reduction of hepatic inflammation [20]. In humans, strong Th17 responses have been linked to tissue injury during atherosclerotic artery disease [21], biliary cirrhosis [22], multiple sclerosis [23], and systemic lupus erythematosus [24], while studies in murine models of experimental autoimmune encephalomyelitis [25] and collagen-induced arthritis [26] have indicated a crucial role for IL-17 in the development of pathology. Similarly, our results suggest that the development or progression of bladder pathology during *S. haematobium* infection might be mediated by Th17 cells.

While Th17 cells have been shown to mediate pathology, Tregs may play a critical role in suppressing immune activation of aggressive T cells [27] through mechanisms that may include IL-10 and/or transforming growth factor  $\beta$  (TGF- $\beta$ ) [28]. In schistosome-infected mice, CD4<sup>+</sup>CD25<sup>+</sup> cells have been identified as a source of IL-10 [29], and *S. mansoni*–infected mice treated with anti-IL-10R and anti-TGF- $\beta$  monoclonal antibody have increased mortality accompanied by an increase in IL-17 production [30], supporting the notion that the balance between Th17 cells and Tregs could be critical in clinical disease outcome in humans.

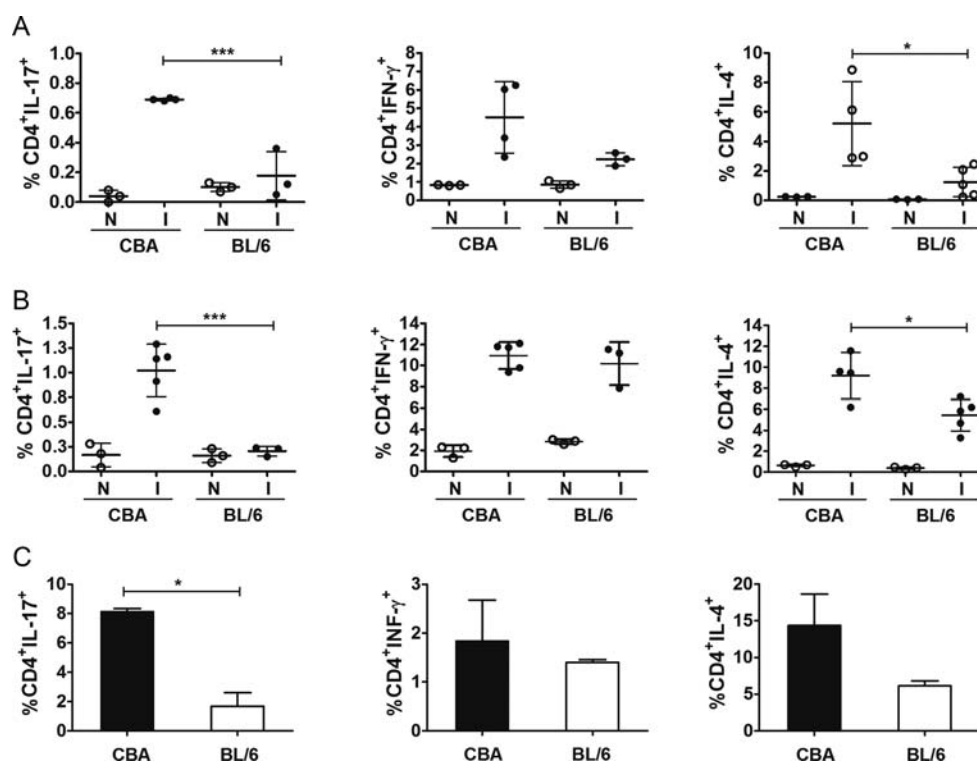
While we found no statistically significant difference in Tregs between the groups, increased ratios of CD4<sup>+</sup>IL-17<sup>+</sup> T



**Figure 4.** Increased numbers of granulocytes in patients with pathology. Scatter plots of median and interquartile ranges of total granulocytes (A), neutrophils (B), and eosinophils (C) in the S–P– (n = 10), S+P– (n = 6), and S+P+ (n = 10) groups are shown. *P* values are based on the Mann–Whitney *U* test. Only statistically significant *P* values are shown. \**P* < .05. Abbreviations: P, pathology; S, *Schistosoma haematobium* infection; –, negative; +, positive.

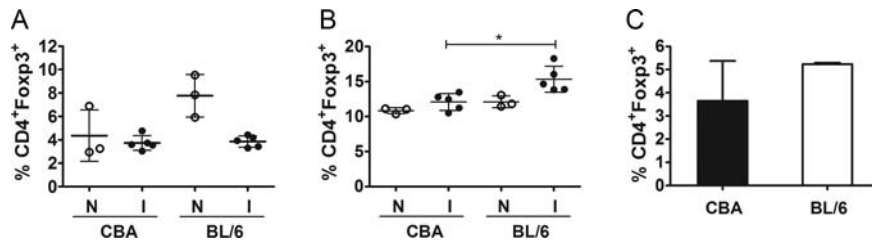
cells to CD4<sup>+</sup>IL-10<sup>+</sup> T cells and of CD4<sup>+</sup>RORγ<sup>+</sup> T cells to CD4<sup>+</sup>FOXP3<sup>+</sup> T cells were found in patients with pathology, compared with those without pathology. The high ratio of Th17 cells to Tregs in patients with pathology seems predominantly due to higher Th17 cell levels. However, we did observe a trend for lower levels of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> T cells in patients with pathology. Although the difference was

not statistically significant, it raises the question of whether more-pronounced differences in Tregs might be seen in organs, as was observed in our murine model, in which the number of Tregs were reduced in organs. Although Th17 and Treg cell development is reported to be mutually exclusive, the expansion of Th17 cells might occur without Treg retraction, explaining the observation that our increased



**Figure 5.** Increased T-helper 17 cells in peripheral blood, spleen, and granulomas from CBA mice. The percentages of interleukin 17 (IL-17)<sup>+</sup>, interferon γ (IFN-γ)<sup>+</sup>, and interleukin 4 (IL-4)<sup>+</sup> CD4<sup>+</sup> T cells in peripheral blood mononuclear cells (PBMCs; A) and spleen cells (B) from uninfected and infected CBA and BL/6 mice are shown. C, Percentages of IL-17<sup>+</sup>, IFNγ<sup>+</sup>, and IL-4<sup>+</sup> CD4<sup>+</sup> cells in granulomas of infected CBA and BL/6 mice are depicted. For PBMCs and spleen cells, data are expressed as values from individual mice and are representative of 3 independent experiments. For granuloma cells, the average of 2 groups of pooled is shown. Data are shown as mean ± SD. *P* values were calculated using 1-way analysis of variance. Only statistically significant *P* values between CBA and BL/6 mice are shown. \**P* < .05, \*\*\**P* < .005. Abbreviations: N, naive; I, *Schistosoma mansoni* infected.





**Figure 6.** Fcpx3<sup>+</sup> cells in peripheral blood, spleen, and granulomas from BL/6 mice. Scatter plots of the percentage of Fcpx3<sup>+</sup>CD4<sup>+</sup> in peripheral blood mononuclear cells (PBMCs; *A*) and spleen cells (*B*) from uninfected and infected CBA and BL/6 mice, as well as the percentage of Fcpx3<sup>+</sup>CD4<sup>+</sup> granuloma cells (*C*) in infected CBA and BL/6 mice are shown. For PBMCs and spleen cells, data are expressed as values from individual mice and are representative of 3 independent experiments. For granuloma cells, the average of 2 groups of pooled granuloma cells is shown. Data are shown as mean  $\pm$  SD. *P* values were calculated using 1-way analysis of variance. Only statistically significant *P* values between CBA and BL/6 mice are shown. \**P* < .05. Abbreviations: N, naive; I, *Schistosoma mansoni* infected.

Th17 cell numbers are not accompanied by a reduced level of Tregs.

In contrast to our findings concerning Th17 cells, the percentage of Th1 and Th2 cells did not differ significantly between *S. haematobium*-infected subjects with and subjects without pathology and, surprisingly, did not differ between infected and uninfected individuals. However, previous exposure of children to schistosomes might explain the Th2 cell response seen even in the absence of current infection [31].

We also found a significant difference in total levels of granulocytes and eosinophils but not when only neutrophils were considered, despite a tendency for higher neutrophil levels in patients with pathology. Given that previous studies have linked neutrophil recruitment with Th17 cell responses [32] and in the context of IL-17-related hepatic infiltration of neutrophils in schistosomiasis [30], our results would suggest peripheral blood neutrophils do not correlate with neutrophil recruitment in inflamed tissues. Although we found no studies that assessed the relationship of neutrophilia with neutrophils in tissues during infection, one study reported that mucosal neutrophils could be detected in neutropenic patients when neutrophils were not present in blood samples [33]. The increased number of eosinophils might reflect the higher activation of immune responses in this group of patients with pathology [34].

One important point to consider is how accurately the profiles found in the peripheral blood mirror those present in the target tissue. While the high number of circulating Th17 cells might reflect their continuous trafficking to the bladder wall, it cannot be assumed that these findings reflect the number of Th17 cells at the site of pathology. However, the murine model supports the notion that the immunological profile of peripheral cells correlates with the response in the tissue, as *S. mansoni*-infected CBA mice with severe pathology not only had significantly higher percentages of CD4<sup>+</sup>IL-17<sup>+</sup> T cells in the periphery as well as in the spleen and granulomas, compared to low-pathology BL/6 mice, they also had higher percentages of CD4<sup>+</sup>IL-4<sup>+</sup> T cells in the periphery and tissues.

An important question to address is whether it is appropriate to extrapolate our results from mouse to human, from *S. mansoni* to *S. haematobium*, and from acute to chronic infection. Murine schistosomiasis has been the most studied experimental model. However, infections involving a single exposure in mice and gradual exposure in humans, a higher infection intensity in the murine model than in humans, and a timing of infection that is mostly undefined in humans may constitute limitations for extrapolating data obtained from mice to humans [35, 36].

In summary, elevated levels of peripheral Th17 cells in patients with urinary tract pathology supports the hypothesis that Th17 cells mediate the development of immunopathology during schistosome infection. By using the murine model, we have further shown that the IL-17 inflammatory environment observed in the peripheral blood reflects the immunopathological response in the tissue. It will now be important to pursue these findings with additional assays, such as functional tests to elucidate the polarization mechanisms and/or differences in signaling pathways that may be responsible for different helper T-cell responses in human patients with and those without pathology.

## Notes

**Acknowledgments.** We thank the population of Pakh and the members of the Association des Jeunes de Pakh for their hospitality and participation in this study. This study would not have been possible without the field workers in Richard Toll, Abdoulaye Yague, Mankeur Diop, Moussa Wade, Ngary Sy, and Boly Diop, who helped with the stool and urine sample collection and did the microscopic analysis. We also thank the medical staff of the Health Center in Richard Toll for their support, the members of the Parasitology Department of Leiden University Medical Center, Alwin van der Ham, Yvonne C. M. Kruijze, and Linda May, as well as Abena S Amoah from Department of Parasitology of Noguchi Memorial Institute for Medical Research of Ghana, for their help.

**Disclaimer.** The funding agencies had no role in experimental design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Financial support.** This work was supported by the European Union's Sixth Framework Program (grant INCO-CT-2006-032405 [for

human studies]) and the US Public Health Service (grant R01-18919 [for mouse studies]).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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