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Schistosome infection is associated with enhanced whole-blood IL-10 secretion in response to cercarial excretory/secretory products

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SUMMARY

Infection of the human host by schistosome parasites follows exposure of skin to free-swimming cercariae and is aided by the release of excretory/secretory (E/S) material, which is rich in proteases and glycoconjugates. This material provides the initial stimulus to cells of the innate immune system. The study presented here is the first to examine human innate/early immune responsiveness to cercarial E/S in subjects from an area co-endemic for Schistosoma mansoni *and* S. haematobium. *We report that in infected participants, stimulation of whole-blood cultures with cercarial E/S material (termed 0–3 hRP) caused the early (within 24 h) release of greater quantities of regulatory IL-10, compared with uninfected controls. Elevated levels of IL-10 but not pro-inflammatory TNFα or IL-8 were most evident in participants co-infected with* S. mansoni *and* S. haematobium *and were accompanied by a higher 0–3 h RP-specific IL-10: TNFα ratio. We also report that glycosylated components within 0–3 h RP appear to be important factors in the stimulation of IL-8, TNFα and IL-10 production by whole-blood cells.*

Keywords *cercariae, excretory/seretory antigens, human disease, IL-10 cytokine, schistosomiasis*

INTRODUCTION

Schistosomiasis remains one of the worlds major parasitic diseases with over 200 million infected people and over 700 million people at risk of infection (1,2). Three major species are known to infect humans: *Schistosoma mansoni* (prevalent in Africa and South America), *S. haematobium* (Africa) and *S. japonicum* (South-east Asia) and can have a significant impact on host morbidity (3). Infection of the human host by these species follows exposure of skin to infective free-swimming cercariae during contact with contaminated freshwater sources. These larvae burrow into the skin, losing their tails in the process, and release the contents of their acetabular glands to aid penetration, thereby providing the initial antigenic stimulus to cells of the innate immune system in the skin (4). The antigenic molecules released from the acetabular glands by transforming cercariae in the first 3 h (termed 0–3 h RP; RP for released product) (5) are rich in proteases (6) and are heavily glycosylated (7). Consequently, this excretory/secretory (E/S) material is likely to contain a variety of ligands for innate immune receptors such as toll-like receptors (TLRs) (8), and C-type lectins (CLRs) including the mannose receptor (9). The innate immune response is critical in shaping the subsequent acquired immune response.

As individuals living in endemic areas are liable to be exposed to infectious cercariae on multiple occasions during domestic, recreational, or occupational water contacts, it has been suggested that repeated exposure to E/S antigens released by invading cercariae may modulate the hosts immune response (5). Indeed, in an experimental murine model, multiple infection with *S. mansoni* cercariae down-modulated $CD4^+$ T-cell responses in the skin-draining lymph nodes (10). Multiple infection also down-regulated the development of egg-specific responses in distant lymphoid tissues and modulated the size of egg-induced granulomas in the liver (10). Therefore, human immune responsiveness to larval E/S material warrants investigation. Unfortunately, human immune responses to cercarial antigens have been infrequently investigated and have been

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restricted to preparations comprising the soluble fraction of whole cercariae (termed CAP or SCAP) (11–15). This preparation is dominated by cytosolic components recovered from the disrupted cercarial bodies and is therefore not reflective of larval E/S material. Analysis of human immune responses specifically to cercarial E/S material is unprecedented.

The study presented here undertook to make an initial analysis of innate/early immune responsiveness to cercarial E/S (i.e. 0–3 h RP) in a cohort of patients from an area endemic for schistosomiasis in northern Senegal. Specifically, the early cytokine response at 24 h of whole-blood (WB) cultures stimulated with 0–3 h RP was examined. The cytokines studied (i.e. IL-8, TNFα and IL-10) were chosen as ones typically released by innate immune cells such as macrophages and monocytes upon activation. Cytokine responses were compared between individuals who did not harbour patent schistosome infection, those infected with *S. mansoni* alone, and those co-infected with *S. mansoni* and *S. haematobium* to investigate whether responsiveness to larval E/S products is influenced by current infection status. We report that cercarial E/S antigens stimulated the release of greater quantities of regulatory IL-10, but not pro-inflammatory TNFα or IL-8, in participants infected with schistosomes compared with uninfected controls.

METHODS

Ethical permission

This study was conducted in 2009 as part of a larger investigation (SCHISTOINIR) examining immune responses in three endemic countries (16), for which approval was obtained by the review board of the Institute of Tropical Medicine in Antwerp, the ethical committee of the Antwerp University Hospital and Le Comité National dEthique de la Recherche en Santé in Dakar, Senegal. Informed and written consent were obtained from all participants; for children, informed consent was obtained from their parents or legal guardant. The community was offered praziquantel (40 mg/kg) and mebendazole (500 mg) treatment after the study according to WHO guidelines (e.g. (17)).

Study population in senegal

The study population was recruited from the village Diokhor Tack (N16·19°; W15·88°). This Wolof community with ~1000 inhabitants is situated on a peninsula in Lac de Guiers in the north of Senegal. To our knowledge, there have been no periodic anthelmintic treatment (e.g. with praziquantel) programmes in this village prior to our study. *S. mansoni* was first introduced into the region in 1988 following construction of the Diama dam and has rapidly spread (18–20). Previously restricted foci of urogenital schistosomiasis in the lower delta have also spread upstream (21). Most communities in this region are co-endemic for *S. mansoni* and *S. haematobium* (22,23). In total, 47 community members were selected from the wider cohort (22) according to infection status giving three study groups: (i) no detectable schistosome infection (uninfected), (ii) single infection with *S. mansoni* (infected) and (iii) co-infection with *S. mansoni* and *S. haematobium* (co-infected). Participants in the three study groups were chosen to have equivalent age ranges and gender distributions.

Parasitology

Schistosome infection status was determined following collection of two stool and two urine samples from each participant as described previously (22,23). Two Kato-Katz slides of two separate samples of faecal material (25 mg, i.e. 4 × 25 mg in total) were examined for eggs of *Schistosoma* species, *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm (24). *S. mansoni* infection intensity for each participant was expressed as the mean number of eggs per gram (epg) of faeces on an individual basis. *S. haematobium* infection intensity for each participant was determined following ultra-filtration of urine (12-μm-pore-size filter; Isopore) and expressed as the number of eggs detected per 10 mL of urine (ep10 mL) calculated from two samples. Participants were classified as infected if they had a schistosome egg count ≥ 1 egg in one or more of their parasitological samples. Ectopic excretion of *S. mansoni* eggs in urine and *S. haematobium* eggs in stool, a phenomenon recently identified in Diokhor Tack community (22), was included in assessment of schistosome infection/co-infection status.

Whole-blood cultures

Samples of whole venous blood (WB) were collected (~6·5 or 13 mL) into heparinized tubes (Sarstedt Monovette, Aktiengesellschaft & Co., Nümbrecht, Germany). Samples were then diluted 1:4 in RPMI 1640 medium (HEPES no L-Glutamine, Gibco) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin 1 mM pyruvate and 2 mM glutamate (Sigma-Aldrich, USA) 5 h ± 30 min after blood drawing. Diluted WB samples were then plated in triplicate at 200 μL/well in 96-well round bottom plates (Nunc) and cultured in the presence or absence of 0–3 h RP or zymosan for 24 h at 37°C under 5% CO_2. The following day, culture supernatants were recovered

and stored at −80°C until analysis by cytokine-specific ELISAs.

Haematology

A further 2-mL venous blood was collected into EDTA tubes (BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The AcT 5diff Cap Pierce Hematology Analyzer (Beckman Coulter®, Suarlée, Belgium) was used to perform the full blood count quantifying numbers leucocytes (lymphocytes, monocytes, eosinophils, basophils and neutrophils); the proportion of each cell type was expressed as the percentage of total leucocytes. Thirty-nine participants provided blood samples for enumeration of leucocytes (uninfected $n = 11$, infected $n = 11$ and co-infected $n = 17$).

Cercarial E/S material and zymosan stimulant

Cercarial E/S material (0–3 h RP) was prepared as previously described (4,8,25) and used as a stimulant of the WB cultures. Alternatively, aliquots of total 0–3 h RP were treated with sodium metaperiodate (smp0–3 h RP), or mock-treated (m0–3 h RP), to disrupt glycan residues (8,26). WB cultures were stimulated with total 0–3 h RP (50 μg/mL), smp0–3 h RP (25 μg/mL), m0–3 h RP (25 μg/mL), the positive control ligand zymosan (50 μg/mL; Sigma-Aldrich, Dorset, UK) or culture medium without antigen (un-stimulated control). All cultures were conducted in the presence of 5 μg/mL polymyxin B (Sigma-Aldrich) to neutralize any potential endotoxin contamination in antigen preparations. Zymosan was chosen as a nonparasite antigen control as it is a heterogeneous mixture of protein–carbohydrate complexes and thus is more comparable to cercarial E/S material than purified bacterial antigens (e.g. LPS).

Cytokine measurement

Cytokine production (IL-8, TNFα and IL-10) in the WB culture supernatants (diluted between 1:2 and 1:10) was measured by specific ELISA kits (TNFα and IL-8, Invitrogen; IL-10, R&D Systems Europe Ltd, Oxford, UK) according to the manufacturers guidelines. Results are given for each patient as mean cytokine production from triplicate wells in response to each stimulant minus the cytokine production for the corresponding WB sample cultured in the absence of stimulant (i.e. medium only).

Statistics

Statistical analyses were conducted using the software package IBM Statistics, version 19. *S. mansoni* infection intensity (log(x + 1)-transformed epg) was compared by gender, age group (5–20 years (children) and ≥20 years (adults)) and infection status (infected and co-infected) tested via ANOVA using sequential sums of squares to account for gender and age before comparison between infection statuses. Age groups were selected according to epidemiological patterns of schistosome infection in the Diokhor Tack community as a whole (22,23). Log (x + 1)-transformed *S. haematobium* ep10 mL was compared by gender and age group via ANOVA for the co-infected group. *S. mansoni* and *S. haematobium* infection intensities were log(x + 1)-transformed to meet parametric assumptions, and the homogeneity of error variances and normality of ANOVA residuals was confirmed using the Levenes test and Shapiro–Wilk test, respectively.

Differences in cytokine concentrations present in antigen-stimulated culture supernatants were compared to those in un-stimulated cultures using the nonparametric paired Wilcoxon signed-rank test. For all subsequent statistical analyses, IL-8, TNFα and IL-10 concentrations present in un-stimulated cultures were subtracted to give stimulus-specific cytokine levels for each individual. The ratio of IL-10: TNFα was calculated from stimulus-specific cytokine levels. As cytokine concentrations, IL-10: TNFα ratios, smp0–3 h RP: m0–3 h RP cytokine ratios, and leucocyte percentages did not meet parametric assumptions, the Mann–Whitney *U*-test and Kruskal–Wallis tests were used to compare between two independent groups and K independent groups, respectively. The Wilcoxon signed-rank test was used for paired comparison of periodate-treated and mock-treated WB culture cytokine production.

RESULTS

Patient details

This study comprised a total of 47 individuals from the Diokhor Tack community aged 6–53 years old, of whom 13 were not infected, 14 infected with *S. mansoni* only and 20 co-infected with *S. mansoni* and *S. haematobium* (Table 1). Only two participants in the co-infected group were also positive for soil-transmitted nematode eggs. *S. mansoni* infection intensity did not significantly differ according to gender ($F_{1,30}$: 1·433, $P = 0·241$), age group ($F_{1,30}$: 1·397, $P = 0·246$) or between infected and co-infected groups ($F_{1,30}$: 2·380, $P = 0·133$). *S. haematobium* infection intensity also did not significantly differ between males and females ($F_{1,\ 17}$: 0·240, $P = 0·631$) or between age groups ($F_{3,17}$: 2·501, $P = 0·132$) in the co-infected group.

Table 1 Age, gender and schistosome infection intensity in the study groups.

	Uninfected	Infected	Co-infected
N	13	14	20
Mean age (range)	33·4 (6–52)	27·9 (7–50)	27·8 (7–53)
Standard deviation	15·1	17·2	3·6
Males, Females	3, 10	6, 8	7, 13
Geometric mean *S. mansoni* eggs/g (range)	–	88·5 (10–1170)	197·2 (10–3470)
95% CI	–	±23·7	±52·7
Geometric mean *S. haematobium* eggs/10 mL (range)	–	–	10·6 (0·5–219·5)
95% CI.	–	–	±2·4

IL-10 production but not IL-8 or TNFα is enhanced in infected individuals in response to cercarial E/S products

To investigate innate/early immune responses to 0–3 h RP, IL-8, TNFα and IL-10 were quantified in whole-blood supernatants 24 h post-stimulation. Levels of all three cytokines were significantly higher in 0–3 h RP-stimulated cultures than in un-stimulated cultures (IL-8 Z: −5·968, $P < 0·001$; TNFα Z: −5·905, $P < 0·001$; IL-10 Z: −5·968, $P < 0·001$) with all 47 participants mounting a detectable cytokine response to 0–3 h RP. Participants also produced higher levels of IL-8, TNFα and IL-10 in response to zymosan than in un-stimulated control cultures (IL-8 Z: −5·968, $P < 0·001$; TNFα Z: −5·841, $P < 0·001$; IL-10 Z: −5·905, $P < 0·001$). Interestingly, stimulus-specific IL-8 and IL-10 levels were higher in response to 0–3 h RP than to an equivalent concentration of zymosan in paired cultures (Wilcoxon signed-rank test, IL-8 Z: −5·661, $P < 0·001$ and IL-10 Z: −4·370, $P < 0·001$), whilst TNFα levels were higher in response to zymosan than to 0–3 h RP (Wilcoxon signed-rank test, Z: −4·529, $P < 0·001$). There was no significant correlation between levels of any of the 0–3 h RP-specific cytokines, and schistosome infection intensity and levels did not differ between age groups (data not shown).

Abundant IL-8 and TNFα were produced by WB cultures from infected, co-infected, and uninfected individuals in response to whole 0–3 h RP and the control stimulant zymosan (Figure 1a and Figure 1b), but there were no significant differences between the infected and uninfected groups (IL-8 Z: −1·213, $P = 0·225$, Figure 1a; TNFα Z: −0·922, $P = 0·357$, Figure 1b) or between the co-infected and uninfected groups (IL-8 Z: −0·663, $P = 0·507$, Figure 1a; TNFα Z: −1·621, $P = 0·110$, Figure 1b). There was also no significant difference in IL-8 or TNFα responses to 0–3 h RP between infected and co-infected subjects (IL-8 Z: −0·717, $P = 0·473$, Figure 1a; TNFα Z: −1·050, $P = 0·294$, Figure 1b).

In contrast to the production of IL-8 and TNFα, 0–3 h RP induced significantly elevated quantities of IL-10 by WB cultures in co-infected subjects (median: 327·4 ng/mL, range: 1124·3) compared with uninfected controls (median: 137·5 ng/mL, range: 486·3; Z: −2·063, $P = 0·039$; Figure 1c). The median concentration of IL-10 production in response to 0–3 h RP was also higher in WB from infected (i.e. only positive for *S. mansoni*) participants (median: 190·7 ng/mL, range: 642·4, Figure 1c) compared with uninfected controls but this trend did not reach statistical significance (Z: −1·504, $P = 0·133$, Figure 1c). There was also no significant difference in 0–3 h RP-specific IL-10 secretion between the infected and co-infected groups (Z: −0·436, $P = 0·451$, Figure 1c). The control stimulant zymosan induced levels of IL-10, which did not significantly differ between the three groups (Figure 1c).

Further analysis of the 0–3 h RP-specific ratio of IL-10 to TNFα revealed that there was a significant increase in the cytokine ratio in response to 0–3 h RP in co-infected subjects (median: 0·039, range: 0·116; Z: −2·800, $P = 0·005$, Figure 2) compared with uninfected subjects (median: 0·016, range: 0·139). There was no significant difference between the zymosan-specific IL-10 to TNFα ratio in the different groups. These observations reinforce the theory that 0–3 h RP has regulatory activity and promotes IL-10 production compared with pro-inflammatory TNFα in schistosome-infected individuals.

Eosinophils are more abundant in infected subjects

As cytokine production is likely to be dependent upon the constituent leucocytes in the WB samples, various leucocyte classes were enumerated as a proportion of the total leucocyte count in the three infection groups (uninfected $n = 11$, *S. mansoni* single infected $n = 11$ and co-infected $n = 17$; Figure 3). Eosinophils were the only leucocyte subset that was significantly affected by infection status (Kruskal–Wallis test, $\chi^2 = 8·375$, $P = 0·015$) with a higher percentage of eosinophils in WB from *S. mansoni*-infected (median: 10·6%, range: 34·2, Z: −2·331, $P = 0·020$) and co-infected participants (median: 12·0%, range: 43·2, Z: −2·658, $P = 0·008$) than in WB collected from uninfected participants (median: 4·7%, range: 20·6). There was no significant difference between the percentage of circulating eosinophils in blood collected from infected and co-infected participants (Z: −0·470, $P = 0·638$). This pattern was also seen in absolute eosinophil counts with higher numbers of eosinophils in infected (median: 690, range: 13·05, Z: −2·185, $P = 0·029$) and co-infected (med-

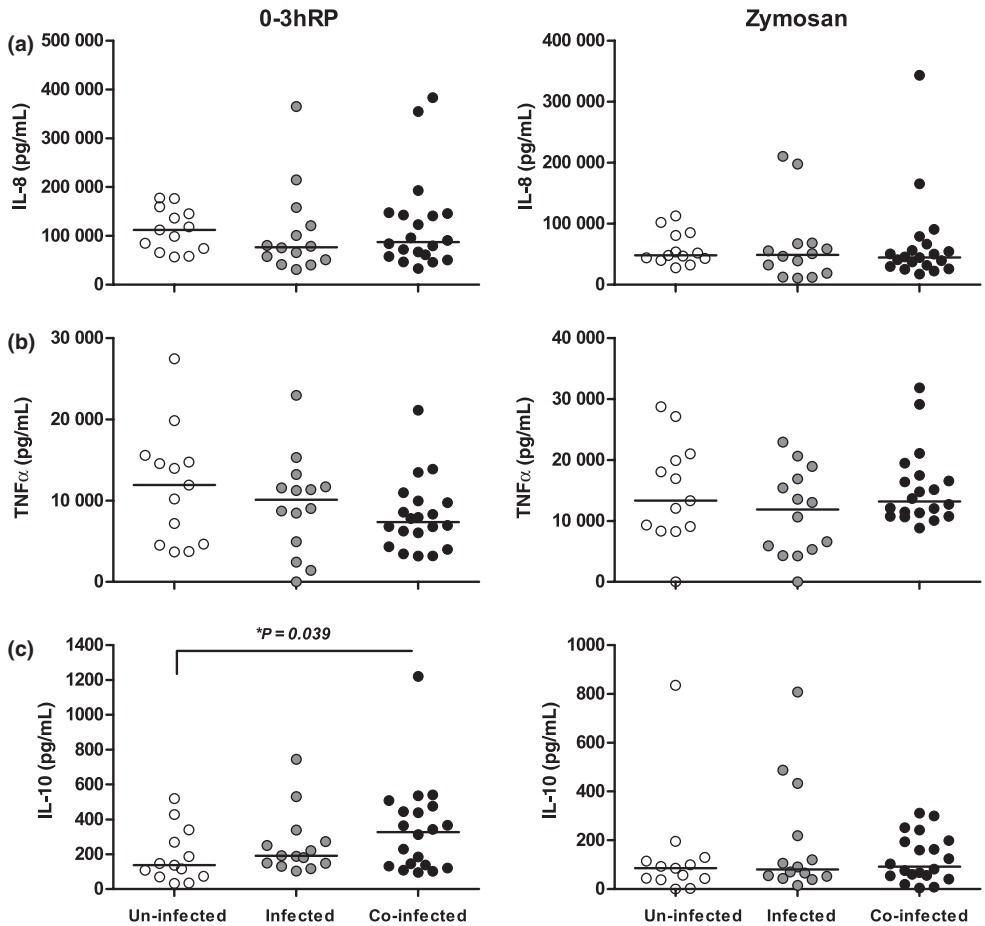

Figure 1 IL-10 but not pro-inflammatory cytokine responses to *S. mansoni* larval secretions are elevated during co-infection relative to uninfected individuals. (a) IL-8, (b) TNFα and (c) IL-10 production in WB cultures. Values are for cytokine production in 0–3 hRP or zymosan-stimulated cultures minus cytokine production in medium control cultures for the same individual. Individual data points are mean values of WB culture supernatants tested in triplicate; statistical significance tested by nonparametric Mann–Whitney *U*-test, * = $P < 0{\cdot}05$, where a *P*-value is not shown and differences were not statistically significant ($P > 0{\cdot}05$). Horizontal bars indicate median.

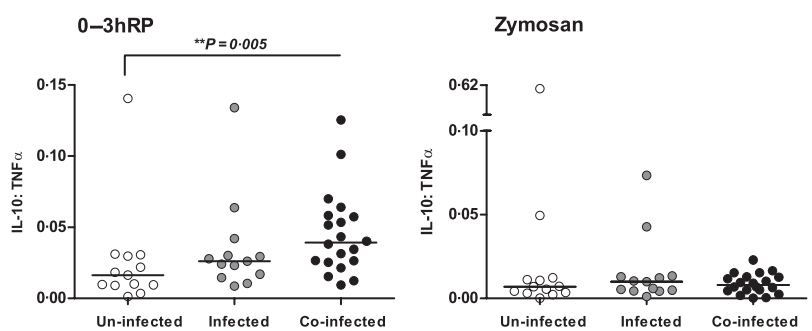

Figure 2 The ratio of IL-10: TNFα production in response to stimulation with 0–3 hRP is elevated during co-infection relative to that in uninfected individuals. The ratio of IL-10: TNFα production by WB from uninfected, *S. mansoni* only and co-infected groups of patients cultured with 0–3 hRP, or zymosan. Significance is shown using nonparametric Mann–Whitney *U*-test; ** $P < 0{\cdot}01$, where a *P*-value is not shown and differences were not statistically significant ($P > 0{\cdot}05$). Kruskal–Wallis analysis: 0–3 hRP $\chi^2 = 8{\cdot}606$, $P = 0{\cdot}014$, zymosan $\chi^2 = 0{\cdot}434$, $P = 0{\cdot}805$, media $\chi^2 = 2{\cdot}493$, $P = 0{\cdot}288$. Horizontal bars indicate median.

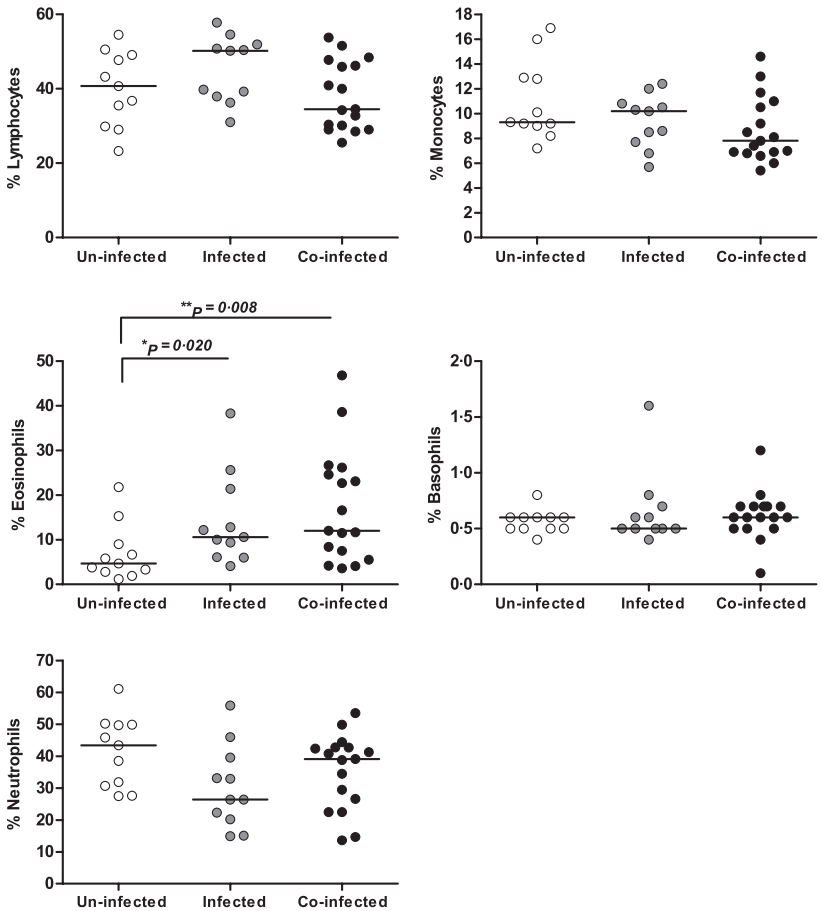

Figure 3 Schistosome infection leads to elevated proportions of circulating eosinophils. Different classes of leucocytes in WB samples were expressed as a proportion of the total leucocyte number. Statistical significance was tested by nonparametric Mann–Whitney *U*-test; $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, where a *P*-value is not shown and differences were not statistically significant ($P > 0{\cdot}05$). Kruskal–Wallis analysis: lymphocytes $\chi^2 = 4{\cdot}432$, $P = 0{\cdot}109$, monocytes $\chi^2 = 4{\cdot}409$, $P = 0{\cdot}110$, eosinophils $\chi^2 = 8{\cdot}375$, $P = 0{\cdot}015$, basophils $\chi^2 = 1{\cdot}403$, $P = 0{\cdot}496$ and neutrophils $\chi^2 = 4{\cdot}515$, $P = 0{\cdot}105$. Horizontal bars indicate median.

ian: 1110, range: 6·21, *Z*: −2·702, $P = 0{\cdot}007$) participants relative to the uninfected group (median: 275, range: 2·21), but no significant difference between the two infected groups (*Z*: −0·753, $P = 0{\cdot}452$). There was no significant difference between the three infection groups in lymphocyte, monocyte, basophil or neutrophil counts.

Cytokine production is reduced in response to de-glycosylated fractions of 0–3 h RP

It has been shown that glycosylated components of 0–3 h RP have an important role for inflammatory cytokine production by murine macrophages (8,9) and polarization of the acquired immune response after infection (9). Here, we investigate the influence of glycans in 0–3 h RP on human cytokine responses to cercarial E/S material in schistosome-infected participants. Consequently, aliquots of total 0–3 h RP were treated with sodium meta-periodate (smp0–3 h RP) to disrupt glycan residues or mock-treated (m0–3 h RP) as the control. This investigation was conducted in 26 participants for whom there was sufficient blood sample volume to conduct the additional WB cultures (infected $n = 11$, co-infected $n = 15$). Using paired WB cultures for these individuals, periodate treatment of 0–3 h RP significantly reduced production of IL-8 (*Z*: −2·354, $P = 0{\cdot}019$), TNFα (*Z*: −4·178, $P < 0{\cdot}001$) and IL-10 (*Z*: −2·134, $P = 0{\cdot}033$) when compared with that produced in response to the mock-treated 0–3 h RP (Figure 4). The ratio of IL-10: TNFα did not differ significantly between periodate-treated and mock-treated control cultures (*Z*: −0·711, $P = 0{\cdot}477$). Furthermore, there was no significant difference between the infected and co-infected groups in the fold change in cytokine secretion between cultures stimulated with m0–3 h RP and smp0–3 h RP (TNFα *Z*:

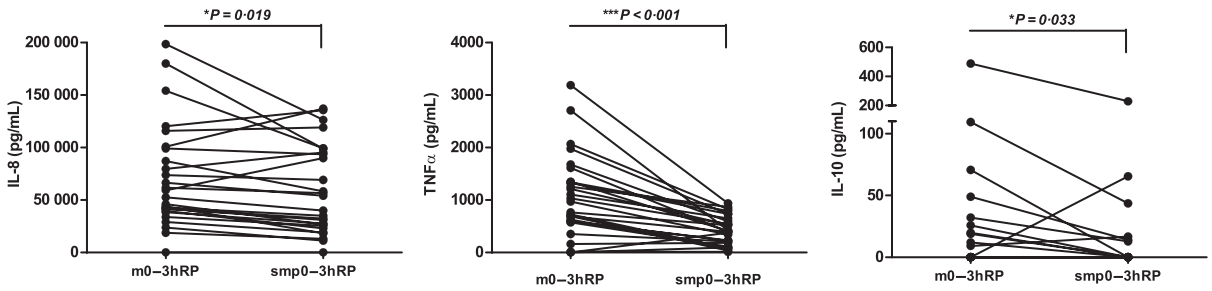

Figure 4 Glycosylated antigens in 0–3 hRP promote pro-inflammatory and regulatory cytokine responses in infected patients. Cytokine production by WB cultures stimulated with either 25 μg/mL mock-treated 0–3 hRP (m0–3 hRP), or 0–3 hRP treated with sodium metaperiodate (smp0–3 hRP). Paired lines link WB cultures for individual patients stimulated with m0–3 hRP and smp0–3 hRP. Statistical significance is shown using nonparametric paired Wilcoxon signed-rank test; $*P < 0{\cdot}05$, $***P < 0{\cdot}001$, where a P-value is not shown and differences were not statistically significant ($P > 0{\cdot}05$).

−0·176, $P = 0{\cdot}861$, IL-8 Z: -0·333, $P = 0{\cdot}739$, IL-10 Z: −1·094, $P = 0{\cdot}274$).

DISCUSSION

In schistosomiasis, cercarial E/S molecules are the first molecules to be presented at the interface with the hosts immune system and are liable to be major agents in stimulating or modulating the innate immune response in the skin (5,27). This is particularly relevant given repeated exposure to cercariae is likely to occur in areas endemic for schistosomiasis. However, it is not known to how many cercariae and on how many occasions any particular individual has been exposed. It is also not known how the innate and acquired immune response in infected humans is affected by such repeated exposure. We have, however, recently shown that cercarial E/S products are major stimulants of murine innate immune cells including dendritic cells and macrophages (4,8,9,25) and that multiple infection of mice with cercariae induces myeloid cells with an alternately-activated phenotype, which down-modulate pathological immune responses to schistosome eggs in the liver (10). Now, we extend studies on cercarial E/S products to the innate/early cytokine response in the natural human host in a schistosome-endemic region.

This study is the first to report on human immune responsiveness to cercarial E/S material, and we show that abundant IL-8, TNFα and IL-10 are produced by WB cells within 24 h of stimulation. Furthermore, compared with uninfected controls, patients co-infected with *S. mansoni* and *S. haematobium* produce significantly greater amounts of immunoregulatory IL-10 when stimulated with 0-3 h RP but not with the control ligand zymosan. Although the sample sizes in each of our three groups (un-infected, *S. mansoni*-infected, and *S. mansoni* and *S. haematobium* co-infected) were limited, this initial investigation showing a significant 0–3 h RP-specific up-regulation of IL-10 in co-infected patients highlights the potential importance of E/S products released from the invasive stage of the parasite in schistosome-infected humans. This provides justification for further larger studies of human immune responsiveness to cercarial E/S antigens.

By collecting WB culture supernatants 24 h after stimulation, we specifically targeted the early production of cytokines released by innate immune cells in WB such as monocytes. We had previously shown using murine macrophages that 0–3 h RP induces abundant IL-10 within 24 h, as well as IL-12p40 and IL-6, and that cytokine production was largely dependent upon functional TLR4 (8). Helminth E/S products, such as 0–3 h RP, are known to have greater stimulatory activity with regard to innate cytokine production than preparations dominated by somatic components (e.g. soluble whole cercariae) (8), which may be more relevant to stimulation of the acquired immune response. We compared the cytokine response to 0–3 h RP with zymosan (derived from the yeast *Saccharomyces*) as a control ligand as like 0–3 h RP, it is biochemically heterogeneous and enriched for glycosylated proteins (9). Zymosan, like 0–3 h RP, also stimulates innate immune cells to drive $CD4^+$ lymphocytes towards a Th2 phenotype (25).

Schistosome infection status at the time of sample collection from individuals in the endemic region was the major factor in determining whether stimulation of WB cells using 0–3 h RP enhances levels of IL-10. Co-infection with *S. mansoni* and *S. haematobium* was associated with the highest production of 0–3 h RP-specific IL-10 relative to uninfected participants. This was not observed in response to the control ligand zymosan or in spontaneous IL-10 production by un-stimulated WB (data not shown). The production of IL-10 can be usefully expressed as ratio compared with production of pro-inflammatory TNFα. As a precedent for this, urinary tract

morbidity in *S. haematobium*-infected patients was linked to a lower ratio of IL-10: TNFα production as part of the acquired immune response (28). Here, we found that the ratio of 0–3 h RP-specific IL-10: TNFα was higher in infected than in uninfected individuals, supporting the hypothesis that cercarial E/S stimulates a regulatory immune phenotype through enhancement of innate/early IL-10 production relative to the production of the pro-inflammatory cytokine TNFα (5,27). The higher ratio of IL-10: TNFα in subjects co-infected with *S. mansoni* and *S. haematobium* also suggests that co-infection may favour immune regulation via IL-10. However, it is also possible that compared to *S. mansoni*, infection with *S. haematobium* is more favourable to IL-10 production, rather than being just a result of co-infection with the two species. Inclusion of a group of patients infected with *S. haematobium* alone would clarify the relative role of the two species. Should co-infected individuals exhibit a more regulated early immune response, this may predispose the host to developing down-regulated response to later stages of parasite development. Indeed, a recent study in the same region of Senegal suggests that co-infection with *S. mansoni* may reduce the risk of *S. haematobium*-associated bladder morbidity (23), and it is possible that IL-10 induced by cercarial E/S material may contribute to this phenomenon. Repeated exposure to cercarial E/S in a schistosome-endemic setting may favour down-regulation of egg-associated pathology in a manner akin to that seen in a murine model of repeated infections (10).

Another possible factor to explain the greater IL-10: TNFα cytokine ratios in co-infected patients might be infection intensity as it has been shown that systemic IL-10 levels are higher in individuals with a greater worm burden (29–31). It might be concluded that co-infected individuals had greater water contact (i.e. increased incidences of exposure leading to infection with both species and/or exposure to a greater number of cercariae) and therefore have higher worm burdens. Indeed, it has previously been shown that *S. mansoni* egg output is greater in co-infected subjects than those infected only with *S. mansoni* in the Diokhor Tack community (22). However, this was not observed in the subcohort of participants in the current study. There was also no correlation between either *S. mansoni* or *S. haematobium* egg output and the production of any of the 0–3 h RP-specific cytokines tested (data not shown). The composition of various leucocyte subsets in WB may also affect the cytokine profile of cultured WB. Although we found no difference in the proportions of neutrophils, monocytes, lymphocytes or basophils, there was a significant increase in the proportion of eosinophils in the WB from both schistosome-infected groups compared with the uninfected control group. Eosinophilia is a common feature of human schistosome infections (32), and eosinophils are a potential source of IL-10 (33,34) but a correlation between elevated eosinophil counts and IL-10 production was not observed. Due to its small size, our study may have lacked statistical power to detect significant correlation between egg output and cytokine production, or leucocyte composition, of WB. Therefore, larger studies will be required to robustly investigate how differences in IL-10 responses to 0–3 h RP may relate to water contact, exposure history, demographic, genetic and immunological characteristics within schistosome-endemic communities.

Finally, glycans from schistosomes are known to have a major role in the stimulation of innate immune responses (35). We previously reported that the cytokine-inducing activity of 0–3 h RP is heat labile (declining at temperatures above 50°C), and glycan dependent (8), with a key role for the mannose receptor (9). Here we show that the production of all 3 cytokines assayed (IL-8, TNFα and IL-10) in WB cultures was reduced after 0–3 h RP was treated with sodium meta-periodate to disrupt the glycosylated moieties. This shows that glycans influence both pro-inflammatory and regulatory cytokine induction in *S. mansoni*-infected humans. However, as molecules released by the mature schistosome egg are also glycosylated (7), and as there is sharing of glycan moieties between different life cycle stages (36), it is possible that innate immune cells that respond to 0–3 h RP (e.g. through C-type lectins such as the macrophage mannose receptor) (9) are also responsive to antigens released by other parasite stages (e.g. the egg)(37), which maintain or down regulate cell responsiveness after initial parasite infection. Therefore, production of cytokines in response to cercarial glycosylated E/S material may be reinforced in response to egg deposition, which may in turn feedback to affect the response to subsequent exposure to cercariae. It is also possible that the Th2-polarized adaptive response dominant after chronic infection in turn influence the ability of innate immune cells to produce IL-10 to cercarial E/S products. It is therefore likely that there will be ongoing communication, or crosstalk, between the innate and adaptive immune systems to regulate reactivity to both cercariae and eggs released by adult worm pairs.

In conclusion, this study is the first to examine immune responses to cercarial E/S antigens, specifically the early production of cytokines indicative of the innate or early adaptive immune response, in human subjects. Our data show that cercarial E/S material induces the production of IL-10 in *S. mansoni*-infected individuals and suggests that cercarial E/S antigens are initial stimulants of a regulated immune phenotype, which is prevalent after repeated and chronic infection with schistosomiasis.

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