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

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Disclosures : None
Competing interests : The authors declare that there are none

Abstract

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 0-3hRP) caused the early (within 24 hours) release of greater quantities of regulatory IL-10, compared to un-infected 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 was accompanied by a higher 0-3hRP-specific IL-10: TNFα ratio. We also report that glycosylated components within 0-3hRP appear to be important factors in the stimulation of IL-8, TNFα and IL-10 production by whole blood cells.

Introduction

Schistosomiasis remains one of the world's 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 hours (termed 0-3hRP; 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 host's 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 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 under took to make an initial analysis of innate/early immune responsiveness to cercarial E/S (i.e. 0-3hRP) in a cohort of patients from an area endemic for schistosomiasis in northern Senegal. Specifically, the early cytokine response at 24 hours of whole blood (WB) cultures stimulated with 0-3hRP 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 harbor 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 to un-infected controls.

Methods

Ethical Permission

This study was conducted in 2009 as part of a larger investigation (SCHISTOINIR) examining immune responses in 3 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 d'Ethique de la Recherche en Santé' in Dakar, Senegal. Informed and written consent was 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 3 study groups; 1) no detectable schistosome infection (un-infected), 2) single infection with *S. mansoni* (infected), and 3) co-infection with *S. mansoni* and *S. haematobium* (co-infected). Participants in the 3 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 x 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. *S. haematobium* infection intensity for each participant was determined following ultra-filtration of urine (12µm pore-size filter; Isopore) and expressed as the number of eggs detected per 10 ml of urine (ep10ml) calculated from 2 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 Tak community (22), was included in assessment of schistosome infection/co-infection status. The prevalence of soil-transmitted helminths in the community was extremely low (2.5%) (22).

Whole blood cultures

Samples of whole venous blood (WB) were collected (~6.5 or 13 ml) into heparinised 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 2mM glutamate (Sigma-Aldrich, USA) 5 hours -/+ 30min 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 0-3hRP or Zymosan for 24 h at 37°C under 5% CO_2. The following day, culture supernatants were recovered and stored at -80ºC until analysis by cytokine-specific ELISAs.

Haematology

A further 2 ml venous blood was collected into EDTA tubes (BD Vacutainer®, USA). The A^{C}●T 5diff Cap Pierce Hematology Analyzer (Beckman Coulter®, USA) was used to perform the full blood count quantifying leucocytes (lymphocytes, monocytes, eosinophils, basophils and neutrophils) and proportions of each cell type were expressed as the percentage of total leucocytes. Thirty nine participants provided blood samples for enumeration of leucocytes (un-infected n=11, infected n=11 and co-infected n=17).

Cercarial E/S material and Zymosan stimulant

Cercarial E/S material (0-3hRP) was prepared as previously described (4, 8, 25), and used as a stimulant of the WB cultures. Alternatively, aliquots of total 0-3hRP were treated with sodium meta-periodate (smp0-3hRP), or 'mock'-treated (m0-3hRP), to disrupt glycan residues (8, 26). WB cultures were stimulated with total 0-3hRP (50 µg/ml), smp0-3hRP (25 µg/ml), m0-3hRP (25 µg/ml), the positive control ligand Zymosan (50 µg/ml; Sigma Aldrich), or culture medium without antigen (un-stimulated control). All cultures were conducted in the presence of 5 µg/ml polymixin B (Sigma Aldrich) in order to neutralize any potential endotoxin contamination in antigen preparations. Zymosan was chosen as a non-parasite antigen control as it is a heterogeneous mix 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) according to the manufacturer's 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') (22)) 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* ep10ml 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 were confirmed using the Levene's 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 non-parametric 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. Since cytokine concentrations, IL-10: TNFα ratios, smp0-3hRP: m0-3hRP ratios, and leucocyte percentages did not meet parametric assumptions, the Mann Whitney U 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 to 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). Two participants in the co-infected group were also positive for soil-transmitted nematode eggs (Table 1). *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.

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 0-3hRP, IL-8, TNFα and IL-10 were quantified in whole blood supernatants 24 hours post-stimulation. Levels of all 3 cytokines were significantly higher in 0-3hRP-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 0-3hRP. 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 0-3hRP 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 0-3hRP (Wilcoxon signed rank test, Z: -4.529, p<0.001). There was no significant correlation between levels of any of the 0-3hRP-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 un-infected individuals in response to whole 0-3hRP and the control stimulant

Zymosan (Fig. 1A and 1B), but there were no significant differences between the infected and un-infected groups (IL-8 Z: -1.213, $p = 0.225$, Fig 1A; TNFα Z: -0.922, $p = 0.357$, Fig 1B) or between the co-infected and un-infected groups (IL-8 Z: -0.663, $p = 0.507$, Fig 1A; TNFα Z: -1.621, $p = 0.110$, Fig 1B). There was also no significant difference in IL-8 or TNFα responses to 0-3hRP between infected and co-infected subjects (IL-8 Z: -0.717, $p = 0.473$, Fig 1A; TNFα Z: -1.050, $p = 0.294$, Fig 1B).

In contrast to the production of IL-8 and TNFα, 0-3hRP induced significantly elevated quantities of IL-10 by WB cultures in co-infected subjects (median: 327.4ng/ml, range: 1124.3) compared with un-infected controls (median: 137.5ng/ml, range: 486.3; Z: -2.063, $p = 0.039$; Fig 1C). The median concentration of IL-10 production in response to 0-3hRP was also higher in WB from infected (i.e. only positive for *S. mansoni*) participants (median: 190.7ng/ml, range: 642.4, Fig 1C) compared to un-infected controls but this trend did not reach statistical significance (Z: -1.504, p= 0.133, Fig. 1C). There was also no significant difference in 0-3hRP-specific IL-10 secretion between the infected and co-infected groups (Z: -0.436, p= 0.451, Fig. 1C). The control stimulant Zymosan induced levels of IL-10 which did not significantly differ between the three groups (Fig. 1C).

Further analysis of the 0-3hRP-specific ratio of IL-10 to TNFα revealed that there was a significant increase in the cytokine ratio in response to 0-3hRP in co-infected subjects (median: 0.039, range: 0.116; Z: -2.800, p = 0.005, Fig. 2) compared to un-infected 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 0-3hRP has 'regulatory' activity and promotes IL-10 production compared to pro-inflammatory TNFα in schistosome--infected individuals.

Eosinophils are more abundant in infected subjects

Since cytokine production is likely to be dependent upon the constituent leucocytes in the WB samples, various leukocyte classes were enumerated as a proportion of the total leucocyte count in the 3 infection groups (un-infected n= 11, *S. mansoni* single infected n=11, and co-infected n= 17; Fig. 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 whole blood 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 whole blood collected from un-infected 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 (median: 1,110 , range: 6.21, Z: -2.702, p=0.007) participants relative to the un-infected group (median: 275, range: 2.21), but no significant difference between the 2 infected groups (Z: -0.753, p=0.452). There was no significant difference between the 3 infection groups in lymphocyte, monocyte, basophil or neutrophil counts.

Cytokine production is reduced in response to de-glycosylated fractions of 0-3hRP

It has been shown that glycosylated components of 0-3hRP have an important role for inflammatory cytokine production by murine macrophages (8, 9), and polarisation of the acquired immune response after infection (9). Here we investigate the influence of glycans in 0-3hRP on human cytokine responses to cercarial E/S material in schistosome infected participants. Consequently, aliquots of total 0-3hRP were treated with sodium meta-periodate (smp0-3hRP) to disrupt glycan residues or 'mock-treated' (m0-3hRP) as the control. This investigation was conducted in 26articipants 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 0-3hRP significantly reduced production of IL-8 (Z: -2.354, p=0.019), TNFα (Z:-4.178, p<0.001) and IL-10 (Z:-2.134, p=0.033) when compared to that produced in response to the mock-treated 0-3hRP (Fig. 4). The ratio of IL-10: TNFα did not differ significantly between periodate-treated and mock-treated control cultures (Z: -0.711, p=0.477). Furthermore, there was no significant difference between the infected and co-infected groups in the fold change in cytokine secretion between cultures stimulated with m0-3hRP and smp0-3hRP (TNFα Z: -0.176, p= 0.861, IL-8 Z: -0.333, p=0.739, IL-10 Z: -1.094, p=0.274).

Discussion

In schistosomiasis, cercarial E/S molecules are the first molecules to be presented at the interface with the host's 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 hours of stimulation. Furthermore, compared to un-infected controls, patients co-infected with *S. mansoni* and *S. haematobium* produce significantly greater amounts of immuno-regulatory IL-10 when stimulated with 0-3hRP 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 0-3hRP-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 24hr 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 0-3hRP

induces abundant IL-10 within 24 hours, 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 0-3hRP, are known to have greater stimulatory activity with regards 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 0-3hRP with Zymosan (derived from the yeast *Saccharomyces*) as a control ligand since like 0-3hRP, it is biochemically heterogeneous and enriched for glycosylated proteins (9). Zymosan, like 0-3hRP, 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 0-3hRP enhances levels of IL-10. Co-infection with *S. mansoni* and *S. haematobium* was associated with the highest production of 0-3hRP-specific IL-10 relative to un-infected 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 to 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 0-3hRP-specific IL-10: TNFα was higher in infected than in un-infected 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 pre-dispose 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 Tak community (22). However, this was not observed in the sub-cohort 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 0-3hRP-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 un-infected 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 0-3hRP 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 0-3hRP is heat labile (declining at temperatures above 50ºC), and glycan

418 dependent (8), with a key role for the mannose receptor (9). Here we show that the
419 production of all 3 cytokines assayed (IL-8, TNFα and IL-10) in WB cultures was
420 reduced after 0-3hRP was treated with sodium meta-periodate to disrupt the
421 glycosylated moieties. This shows that glycans influence both pro-inflammatory and
422 regulatory cytokine induction in *S. mansoni*-infected humans. However, as molecules
423 released by the mature schistosome egg are also glycosylated (7), and as there is
424 sharing of glycan moieties between different life cycle stages (36), it is possible that
425 innate immune cells that respond to 0-3hRP (for example through C-type lectins
426 such as the macrophage mannose receptor)(9) are also responsive to antigens
427 released by other parasite stages (*e.g.* the egg)(37) which maintain, or down
428 regulate cell responsiveness after initial parasite infection. Therefore, production of
429 cytokines in response to cercarial glycosylated E/S material may be reinforced in
430 response to egg deposition, which may in turn feedback to affect the response to
431 subsequent exposure to cercariae. Since in the chronic stage of schistosome
432 infection is dominated by a Th2-polarised adaptive immune response to egg
433 antigens, this may influence the ability of innate immune cells to produce IL-10 to
434 cercarial E/S products. It is therefore likely that there will be on-going
435 communication, or crosstalk, between the innate and adaptive immune systems to
436 regulate reactivity to both cercariae, and eggs released by adult worm pairs.
437
438 In conclusion, this study is the first to examine immune responses to cercarial E/S
439 antigens, specifically the early production of cytokines indicative of the innate or
440 early adaptive immune response, in human subjects. Our data shows that cercarial
441 E/S material induces the production of IL-10 in *S. mansoni*-infected individuals, and
442 suggests that cercarial E/S antigens are initial stimulants of a 'regulated' immune
443 phenotype which is prevalent after repeated and chronic infection with
444 schistosomiasis.
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Acknowledgements:

We gratefully thank the population of Diokhor Tack and the village chief, Daoure Mbaye, 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 and Ngary Sy, who assisted in the blood sample collection and microscopic analysis. We would also like to thank the medical and technical staff of the Health Centre in Richard Toll for their support. The authors would also like to thank Ann Bamford for help in preparation of antigen material. This study was supported by The European Union (EU INCO-CT-2006-032405 to APM, SM, and K P). JDT, LM, PD, MM, TND performed the research; JDT, LM, designed the research study; CDB, JDT, PD, APM analysed the data; APM wrote the paper, CDB, LM, JDT, KP, MM contributed to drafts of the paper: .APM, KP SM secured funding for the study.

Figures

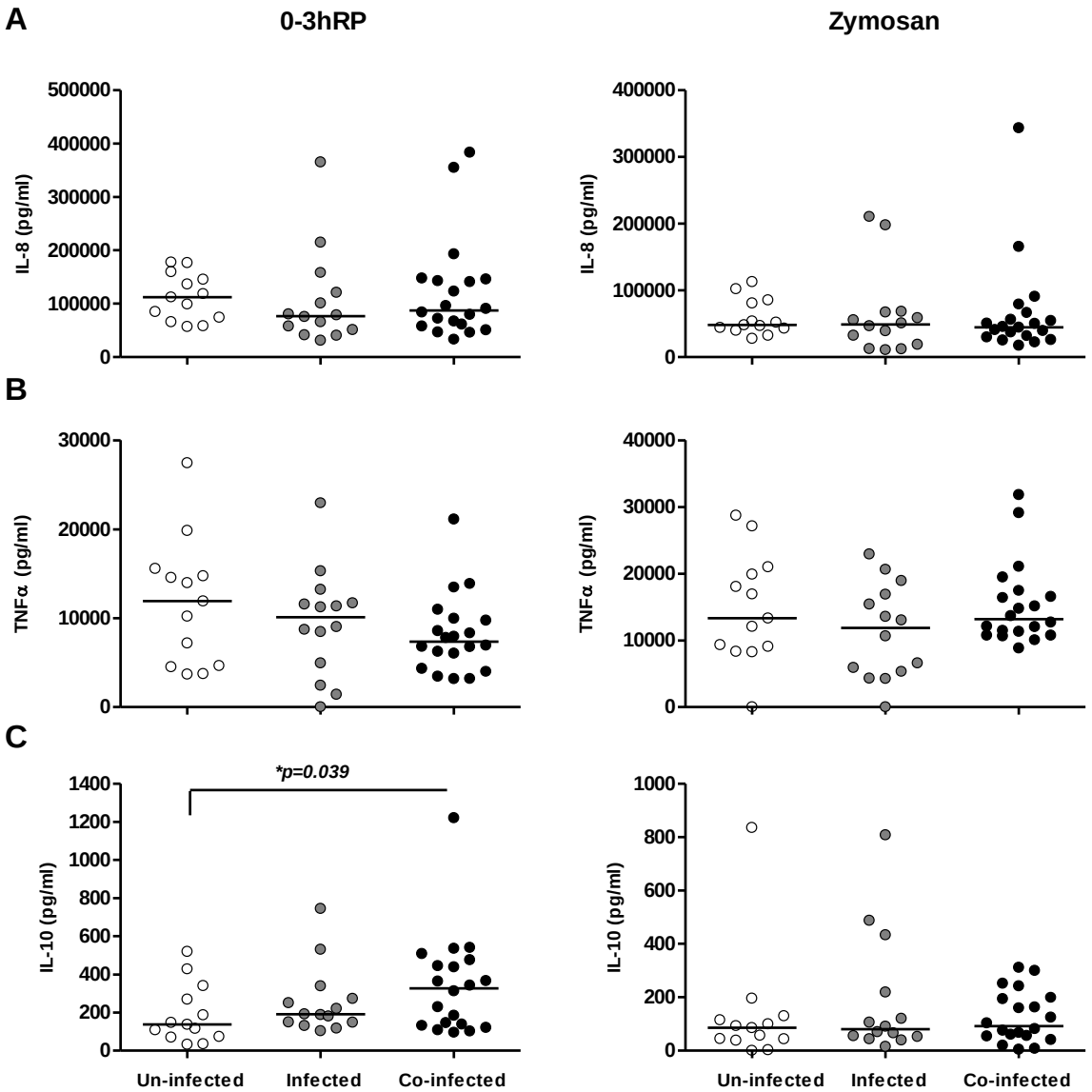


Figure 1

IL-10 but not pro-inflammatory cytokine responses to *S. mansoni* larval secretions are elevated during co-infection relative to un-infected individuals. **A.** IL-8, **B.**TNFα and **C**. IL-10 production in WB cultures. Values are for cytokine production in 0-3hRP-, 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 non-parametric Mann Whitney U, * = p <0.05, where a p-value is not shown differences were not statistically significant (p >0.05). Horizontal bars indicate median.

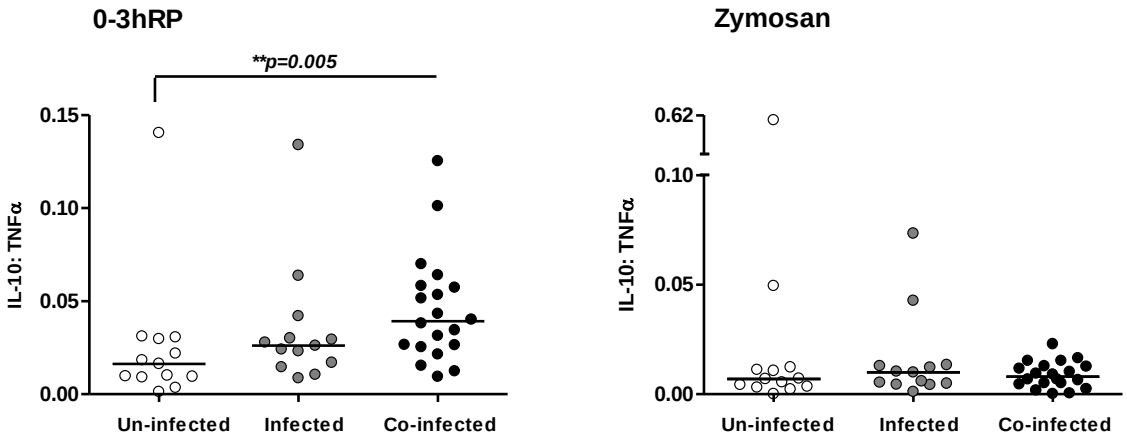
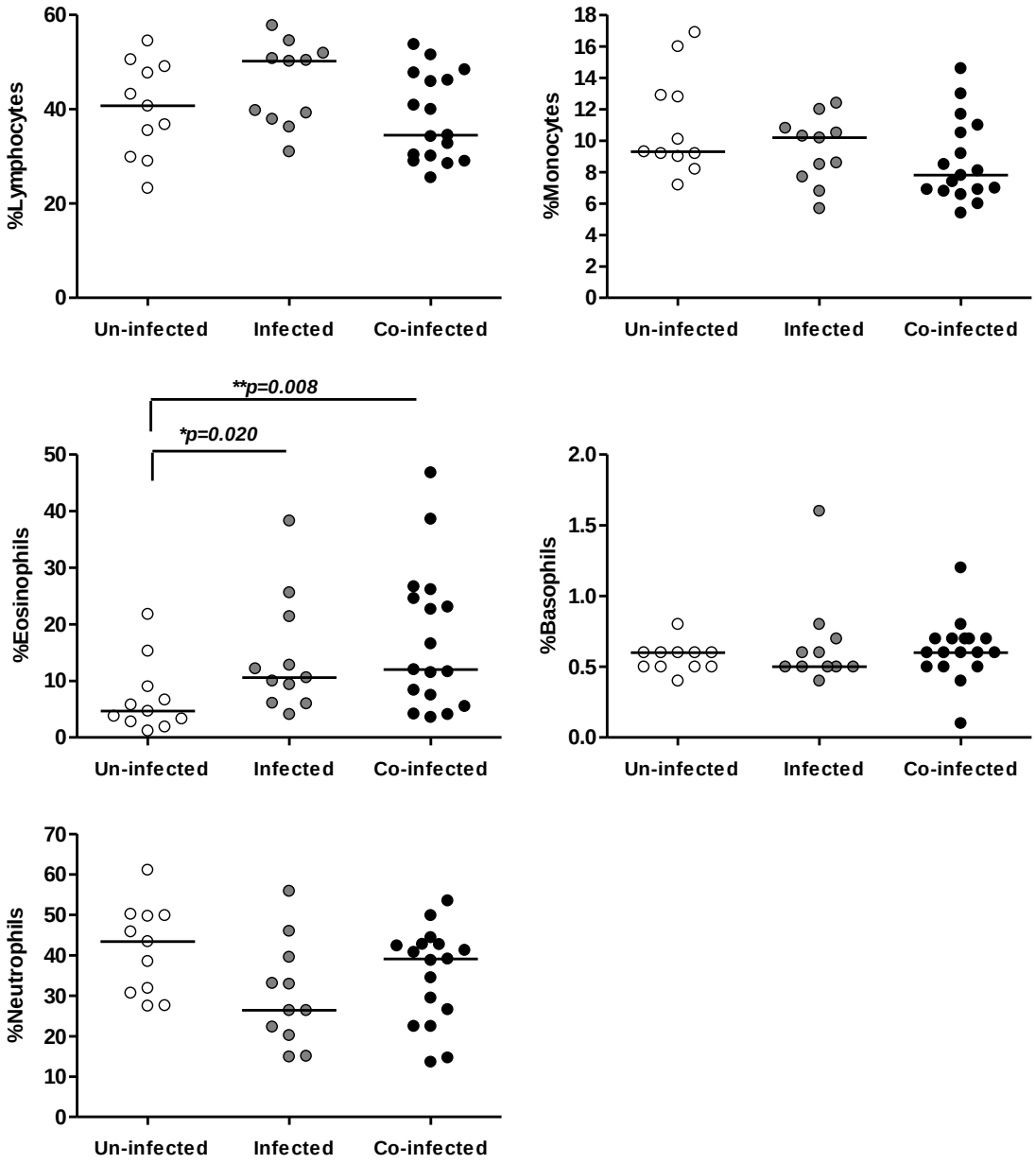


Figure 2

The ratio of IL-10: TNFα production in response to stimulation with 0-3hRP is elevated during co-infection relative to that in un-infected individuals.

The ratio of IL-10: TNFα production by WB from un-infected, *S. mansoni* only and co-infected groups of patients cultured with 0-3hRP, or Zymosan. Significance is shown using non-parametric Mann Whitney U; ** $p<0.01$, where a p-value is not shown differences were not statistically significant ($p>0.05$). Kruskal-Wallis analysis: 0-3hRP $Chi^2 = 8.606$, $p= 0.014$, Zymosan $Chi^2= 0.434$, $p= 0.805$, media $Chi^2= 2.493$, $p= 0.288$. Horizontal bars indicate median.



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510

511 **Figure 3**

512 **Schistosome infection leads to elevated proportions of circulating eosinophils.**
513 Different classes of leucocytes in WB samples were expressed as a proportion of the
514 total leucocyte number. Statistical significance was tested by non-parametric Mann
515 Whitney U; * p <0.05, **p<0.01, where a p-value is not shown differences were not
516 statistically significant (p >0.05). Kruskal-Wallis analysis: lymphocytes Chi^2= 4.432,
517 p=0.109, monocytes Chi^2 =4.409, p=0.110, eosinophils Chi^2 =8.375, p=0.015,
518 basophils Chi^2= 1.403, p= 0.496, and neutrophils Chi^2= 4.515, p=0.105. Horizontal
519 bars indicate median.

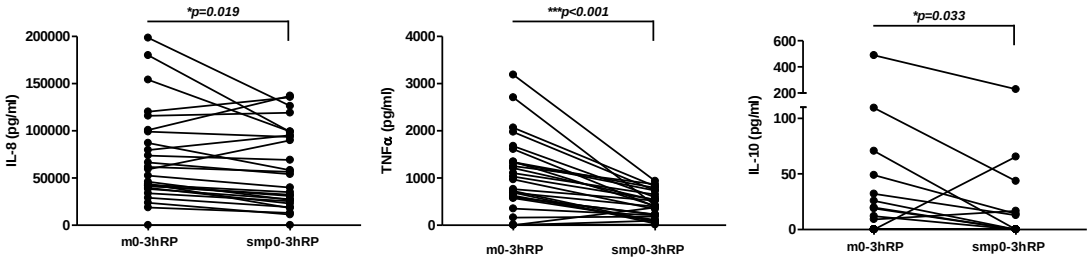

Figure 4

Glycosylated antigens in 0-3hRP promote pro-inflammatory and regulatory cytokine responses in infected patients. Cytokine production by WB cultures stimulated with either 25µg/ml 'mock'-treated 0-3hRP (m0-3hRP), or 0-3hRP treated with sodium meta-periodate (smp0-3hRP). Paired lines link WB cultures for individual patients stimulated with m0-3hRP and smp0-3hRP. Statistical significance is shown using non-parametric paired Wilcoxon test; *p<0.05, ***p<0.001, where a p-value is not shown differences were not statistically significant (p >0.05).

547 **Table 1**

548 **Age, gender and schistosome infection intensity in the study groups.**

	Un-infected	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% C.I.	-	+/- 23.7	+/- 52.7
Geometric mean *S. haematobium* eggs/10ml (range)	-	-	10.6 (0.5 - 219.5)
95% C.I.	-	-	+/- 2.4
No. participant with nematode infection (species)	0	0	2 (*Ascaris spp.)*

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