Exposure, infection, systemic cytokine levels and antibody responses in young children concurrently exposed to schistosomiasis and malaria

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SUMMARY

Despite the overlapping distribution of *Schistosoma haematobium* and *Plasmodium falciparum* infections, few studies have investigated early immune responses to both parasites in young children resident in areas co-endemic for the parasites. This study measures infection levels of both parasites and relates them to exposure and immune responses in young children. Levels of IgM, IgE, IgG4 directed against schistosome cercariae, egg and adult worm and IgM, IgG directed against *P. falciparum* schizonts and the merozoite surface proteins 1 and 2 together with the cytokines IFN-γ, IL-4, IL-5, IL-10 and TNF-α were measured by ELISA in 95 Zimbabwean children aged 1–5 years. Schistosome infection prevalence was 14·7% and that of *Plasmodium* infection was 0% in the children. 43. 4% of the children showed immunological evidence of exposure to schistosome parasites and 13% showed immunological evidence of exposure to *Plasmodium* parasites. Schistosome–specific responses, indicative of exposure to parasite antigens, were positively associated with cercariae-specific IgE responses, while *Plasmodium*-specific responses, indicative of exposure to parasite antigens, were negatively associated with responses associated with protective immunity against *Plasmodium*. There was no significant association between schistosome-specific and *Plasmodium*-specific responses. Systemic cytokine levels rose with age as well as with schistosome infection and exposure. Overall the results show that (1) significantly more children are exposed to schistosome and *Plasmodium* infection than those currently infected and; (2) the development of protective acquired immunity commences in early childhood, although its effects on infection levels and pathology may take many years to become apparent.

Key words: Young children, schistosome, *Plasmodium*, co-infection, immunity, antibody, cytokine, human.

INTRODUCTION

There is considerable overlap in the geographical distribution of the two human parasites, *Schistosoma haematobium* and *Plasmodium falciparum,* and the prevalences of both parasites in exposed individuals rise with age, peaking in childhood. Schistosome infection in children below 5 years of age had, until recently, largely been ignored as a result of two previous misconceptions; (1) that such young children are not sufficiently exposed to water containing viable schistosome cercariae to acquire significant levels of infection, and (2) that low levels of infection carried by pre-school children did not translate into severe morbidity (Stothard and Gabrielli, 2007). With the growing evidence that children aged 5 years and below do carry significant levels of schistosome infection (Mafiana *et al*. 2003; Sousa-Figueiredo *et al*. 2008; Uneke and Egede, 2009; Garba *et al*. 2010), and morbidity (Garba *et al*. 2010) and the indication that this same age group can suffer from severe malaria (Oduro *et al*. 2007), there is a need to understand the interaction between the two parasites in these young children in terms of disease aetiology and impact on child health and development. The pathophysiology of both parasitic infections is immune-mediated, such as cerebral malaria or schistosome granuloma and fibrosis (Mott and Chen, 1989; Maitland and Marsh, 2004). However co-infection is known to modulate immune responses, potentially altering the pathophysiological and immunological profiles of disease (Booth *et al*. 2004*b*,*c*). To date there is a

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paucity of studies describing the burden of *Schistosoma haematobium* infection in children aged 5 years and below and even fewer studies characterizing the early schistosome specific immune responses in these children. There are relatively more studies of *Plasmodium*-specific immune responses in young children but fewer studies characterizing schistosome and *Plasmodium*-specific responses in children concurrently exposed to both parasites.

Investigating the earliest immune responses resulting from exposure to schistosome and *Plasmodium* antigens in childhood not only informs on the nature and development of pathological/protective responses, but also on the phenotype of systemic immune responses at this young age. Therefore we have investigated the relationship between exposure to both parasites and the development of parasite-specific antibody responses. The study focused on immune responses indicative of recent exposure to infection as well as immune responses associated with resistance to infection/re-infection. Parasite-specific IgM responses are associated with recent exposure to parasites and several studies have shown this to be true for both *Plasmodium* and schistosome parasites (Mutapi *et al*. 1997; Ndhlovu and Woolhouse, 1996; Naus *et al*. 2003*a*). In *Plasmodium* infections, IgG1 and IgG3 antibody sub-classes are associated with protection against the merozoite surface proteins (MSP) antigens (Bouharoun-Tayoun and Druihle, 1992; Cavanagh *et al*. 2004). Studies on *S. haematobium* indicate that the balance between adult schistosome-specific IgE and IgG4 is one of the key indicators of the development of protective immunity to infection (Hagan *et al*. 1991) while anti-cercariae IgE and IgG4 responses are associated with the hypersensitivity reaction causing cercarial dermatitis (Kourilova *et al*. 2004; Lichtenbergova *et al*. 2008). Anti-egg IgG4 responses have been associated with pathology in *S. mansoni* infected Brazilians (Silveira *et al*. 2002) and anti-egg IgE has been associated with immunity to re-infection with *S. japonicum* (Zhang *et al*. 1997). Therefore, this study focused on total IgM IgG responses against crude *Plasmodium* schizont antigen (to determine exposure to *P. falciparum* parasites), IgM against schistosome antigens (to determine exposure to *S. haematobium* infections), IgG responses against two *P. falciparum* vaccine candidates, merozoite surface protein (MSP)-1 and MSP-2 we have previously reported on from Zimbabwean populations (Reilly *et al*. 2008) and anti-schistosome IgE and IgG4 as indicators of the development of putatively protective acquired immunity and as risk factors for immunopathology.

Cytokines contribute both to infection-related pathological processes and the development of protective immunity to these parasites (Booth *et al*. 2004*a*; Hunt and Grau, 2003). Interleukin (IL)-10 and interferon-gamma (IFN-γ) are involved in isotype switching to protective IgG sub-classes in

Plasmodium infections (Garraud *et al*. 2003), while IL-4, IL-5 and IL-10 appear to be important for the development of resistance to schistosome infection (Pearce and MacDonald, 2002). There is a growing body of evidence suggesting that there is a significant interaction in the development of protective immunity and pathology in individuals co-infected with these parasites (Arinola, 2005; Diallo *et al*. 2010; Remoue *et al*. 2003; Wilson *et al*. 2008), but there are few studies in young children. A study in Senegal compared systemic cytokine levels in children aged 7–15 years to those in adults aged over 30 years and showed that children co-infected by *S. haematobium* had higher levels of IFN-γ and sTNF-RII compared to children infected only by *P. falciparum*. In contrast, co-infected adults showed a significant increase in IFN-γ, IL-10, TGF-β, sTNF-RI and sTNF-RII, RANTES and the IL-10/TNF-α ratio (Diallo *et al*. 2004). The role of childhood infections in determining future responses mounted to self and non-self antigens is an area of active interest (van den Biggelaar and Holt, 2010).

For this study, systemic cytokines indicative of CD4 + T cell-mediated T helper (Th)1 and Th2-type responses were selected for investigation, as these are amongst the main immunological correlates of pathology and protective immunity to *Plasmodium* and schistosome infections (Pearce and MacDonald, 2002; Maizels and Yazdanbakhsh, 2003; Hunt and Grau, 2003; Booth *et al*. 2004*a*; Lyke *et al*. 2004; Awandare *et al*. 2006). IFN-γ, and TNF-α are markers of Th1 responses, IL-4 and IL-5 are markers for Th2 responses, and IL-10, originally classified as both a Th1 and Th2 cytokine in humans (Del Prete *et al*. 1993), is now also seen as a marker for immunomodulation and regulatory responses (van den Biggelaar *et al*. 2000; Hesse *et al*. 2004; McKee and Pearce, 2004). Regulatory responses appear to play an important role in limiting immunopathology in single-species infections (Malaquias *et al*. 1997; Hunt and Grau, 2003; Booth *et al*. 2004*a*) and may also be important in malaria-schistosome co-infection.

The aim of this study was to determine the level of infection with, and exposure to *S. haematobium* and *P. falciparum* in children aged 1–5 years resident in villages endemic for both parasites and relate this to the development of parasite-specific immune responses and levels of systemic cytokines.

MATERIALS AND METHODS

Study area

The study was conducted in two villages, Magaya and Chitate, in the Mashonaland East Province of Zimbabwe (31°90′E; 17°63′S) where *S. haematobium* is endemic. The participants are involved in ongoing studies on the control and immuno-epidemiology of

human schistosomiasis. The main activity in these villages is subsistence farming and human water contact is frequent with at least 4 contacts/person/ week (assessed by questionnaire) due to insufficient safe water and sanitation facilities. The rivers in the two villages differ in their temporal patterns; those in Magaya are mostly perennial while those in Chitate are seasonal, leading to different schistosome transmission dynamics. Older children are exposed to infective water actively while younger children are also exposed passively (e.g. sitting in a container with infective water) (Stothard and Gabrielli, 2007) as indicted by questionnaire responses of their parents/ guardians. *Plasmodium falciparum* is the predominant species of malaria in Zimbabwe (Taylor and Mutambu, 1986) where malaria transmission is largely unstable in nature. Approximately 5·5 million people out of a total population of 12·7 million live in malarious areas (Stanley Midzi *et al*. 2004). Out of the 56 districts in Zimbabwe, malaria transmission occurs in 42. In 2002, a revised stratification based on a national parasite prevalence survey, Health Management Information Systems (HMIS) data, entomological data and expert opinion classified our study area under the sporadic transmission regions with low transmission (Mabaso *et al*. 2005, 2006) meaning that this is a mesoendemic area for *Plasmodium* transmission and malaria (Mharakurwa *et al*. 2004). The peak *P. falciparum* transmission occurs from February to May (Mharakurwa *et al*. 2004) and the annual incidence of malaria in the area is 1–10 cases/1000 people (Mabaso *et al*. 2006; Hay *et al*. 2009). The study was conducted in February.

Ethical statement

Permission to conduct the study in the region was obtained from the Provincial Medical Director. Institutional and ethical approval was received from the University of Zimbabwe and the Medical Research Council of Zimbabwe respectively. At the beginning of the study, parents and guardians of participating children had the aims and procedures of the project explained fully in the local language, Shona, and written consent was obtained from participants' parents/guardian before enrolment into the study. After collection of all samples, all participants and their parents/guardians were offered anthelmintic treatment with the recommended dose of praziquantel (40 mg/kg of body weight).

Sample collection and inclusion criteria

Stool and urine specimens were collected from each participant on 3 consecutive days and examined microscopically for *S. haematobium, S. mansoni* and geo-helminths following standard procedures (Katz *et al*. 1972; Mott, 1983). The formol-ether

concentration method was performed as previously described (Cheesbrough, 1998; Midzi *et al*. 2008) on a random sample of the stool samples to confirm results obtained by the Kato-Katz technique. For infants, samples were collected overnight if it was not possible to collect a sample on the spot. A maximum of 5 ml of venous blood was collected and processed as previously described (Mutapi *et al*. 2008) for *Plasmodium* detection cytokine and antibody assays. Thick and thin smear slides were prepared per participant upon blood collection for the microscopic detection of *Plasmodium* parasites. Results from these examinations were confirmed using the rapid Paracheck dipstick (Orchid Biomedical Systems). Plasma samples were stored at $-20\,^{\circ}\mathrm{C}$ and freighted frozen to Edinburgh where they were thawed for the assays described below.

In order to be included in the study, participants had to meet all the following criteria: (1) have been resident in the study area since birth; (2) provided at least two urine and two stool samples on consecutive days; (3) be negative for intestinal helminths including *S. mansoni* (no-one was excluded on this criteria as everyone was negative for these infections as is reported in other parts of Zimbabwe (Midzi *et al*. 2010) and (4) have given a blood sample for the collection of sera. A total of 95 (54 from Magaya and 41 from Chitate) participants aged 1–5 years met these criteria and formed our study population.

Parasite antigens

Lyophilized soluble *S. haematobium* adult worm antigen (WWH), cercariae antigen (CAP) and soluble egg antigen (SEA) were obtained from the Theodor Bilharz Institute (Egypt) and reconstituted as described elsewhere (Mutapi *et al*. 2005). The parasite strain is one used for our previous immuno-epidemiology studies (Milner *et al*. 2010; Mutapi *et al*. 2008; Reilly *et al*. 2008). The *P. falciparum* antigens were those previously used in a study in Zimbabwe (Reilly *et al*. 2008) and included crude schizont antigen and the recombinant merozoite surface protein (MSP) antigens prepared in *Escherichia coli*, MSP-1_{19} antigen also known as p190, gp195, (Burghaus and Holder, 1994; Holder *et al*. 1994) and two full-length recombinant MSP-2 antigens, namely CH150/9 (5/6) and Dd2 (13/14) (Metzger *et al*. 2003). MSP-2 has 2 serotypes: CH150/9 5/6 is taken from serotype A (3D7-like), and Dd2 belongs to serotype B (FC27-like).

Antibody assays

IgM, IgE and IgG4 specific for schistosome antigens from the three life stages (cercariae, egg and adult) were measured by indirect enzyme linked immunosorbent assays (ELISA) as previously described

(Mutapi *et al*. 1997). *P. falciparum*-specific responses (IgM and IgG) were also measured by ELISA as previously described (Aribot *et al*. 1996; Oeuvray *et al*. 1994; Theisen *et al*. 1998). Briefly, ELISAs were conducted in duplicate for each sample using ELISA plates (Nunc-Immulon, Denmark) that were coated with 50 μl/well of 50 ng/ml antigen for recombinant antigens and 1 μg/ml for both crude antigens in 60 mM carbonate-bicarbonate buffer (pH 9·6) and incubated overnight at 4 °C. Plates were blocked with 200 μl/well of skimmed milk (5% milk in phosphate buffered saline (PBS)/0·03% Tween 20) for 1 hr and washed three times in PBS/Tween 20, which was used for all washes. 100 μl of serum was added to each well at 1:100 dilution for all assays; plates were incubated at 37 °C for 2 hours and then washed three times. 100 μl of subclass-specific monoclonal antibody (IgM for crude antigens and IgG for both crude and recombinant antigens, both from Dako UK) were added at 1:1000 dilution for crude antigens and 1:2000 dilution for recombinant antigens. Plates were incubated overnight at 4 °C, washed six times and 50 μl of ABTS substrate solution (KPL, Canada) was added, before the absorbance was read at 405 nm. Negative controls from 5 age-matched schistosome and *Plasmodium* naïve European donors (aged 1–5 years old) were included in duplicate on each of the IgM plates to indicate cut-off points for reactivity, while 2 negative controls were included on the rest of the plates.

The anti-schistosome egg IgM antibodies were also used diagnostically based on exposure to egg antigens as recently reported in a study in preschool children exposed to *S. mansoni* infection in Uganda (Stothard *et al*. 2011). Anti-cercariae IgM and anti-schizont IgM were used to indicate recent exposure to schistosome cercariae and *Plasmodium* schizonts as previously described for schistosomiasis (Woolhouse *et al*. 2000) and *Plasmodium* respectively (Naus *et al*. 2003*b*). To determine if children showed recent exposure to parasites (schistosome cercariae, worm or egg, or *Plasmodium* schizonts), levels of the parasite-specific antibodies (optical densities) had to be greater than the mean + 2 standard deviations of the negative controls.

Cytokine assays

Circulating levels of IFN-γ, IL-4, IL-5, IL-10 and TNF-α in plasma samples were conducted using capture ELISA with antibody capture/detection pairs and standards from BD Biosciences following previously published protocols (Joseph *et al*. 2004; Milner *et al*. 2010) and manufacturer's guidelines. All assays were conducted in duplicate. People with cytokine levels above zero ng/ml (after the subtraction of the blank control) were denoted positive for the cytokine while those with concentrations of zero ng/ml or below were designated as having no detectable levels of the cytokine.

Statistical analyses

Infection prevalence was compared between villages by Chi-squared test. For analyses on antibody levels the continuous optical density data were used to ensure that the early dynamics of the parasite-specific immune responses could be captured even in children producing low levels of the antibodies. Thus levels of antibodies directed against the different schistosome crude antigens were compared using the non parametric Kruskal Wallis test (data did not satisfy the assumptions of parametric tests) while antibody responses against *P. falciparum* crude antigens were compared using the Mann-Whitney Test. The effects of host sex (categorical, male and female), age (3 categories, 1–3 years, 3.1 yrs–4 years and 4.1–5 years to give sufficiently large sample sizes for statistical analyses), village (categorical, Magaya and Chitate) and schistosome infection level either as a categorical variable (egg positive or egg negative) or continuous variable (log (x + 1) transformed) on antibody level (square root transformed) were tested using an analysis of variance. Sequential sums of squares were used to account for variation due to all other factors before testing for the effects of schistosome infection level and the interaction between village and age group (to determine the presence of a peak shift) (Mutapi *et al*. 1997).

Given that the study measured several immune variables, i.e. 9 schistosome-specific responses, 5 *Plasmodium*-specific responses and 5 cytokines, reducing the number of variables was used as a means of identifying dynamics of exposure and the development of parasite-specific acquired immunity. Thus all the parasite-specific antibody variables and cytokine variables were reduced into uncorrelated variables by factor analysis, a standard technique for reducing multivariate data to its main independent features, by transforming several correlated variables into fewer uncorrelated variables called principal components (Sokal and Rohlf, 1995). The components are extracted according to the amount of variation in the data they explain, so the first component explains the most variation and each subsequent component is included if it explains a significant amount of the remaining variation within the data (Sokal and Rohlf, 1995). Principal components with eigenvalues greater than one were extracted by regression analysis and an extracted component was considered reliable if it had one or more of the original variables with factor loadings ⩾ 6 or ⩽ − 6 and below. Principal component regression factor scores for each participant were used in analyses of variance to determine their relationship with host factors (sex, age group, village, schistosome infection intensity and the

Table 1. Schistosome infection level in the study population

	Magaya (High infection area)			Chitate (low infection area)		
	Infection intensity range (sample size)	Infection intensity mean* (SEM)**	Infection prevalence	Infection intensity range (sample size)	Infection intensity mean (SEM)	Infection prevalence
1–3 years old	0–22 (7)	3·14 (3·14)	14·3%	0 (11)	0	0
3.1–4 years old	0–458 (18)	25·44 (25·44)	6·6%	0 (19)	0	0
4.1–5 years old	0–268 (31)	16·26 (9·09)	35·5%	0–1 (9)	0·04 (0·04)	11·1
Total	0–458 (56)	17·57 (9·50)	23·2%	0–1 (39)	0·01 (0·01)	2·6

* = arithmetic mean.
** SEM = Standard error of the mean.

village × age group interaction). After exploratory plots, one tailed Pearson correction analyses was conducted to determine the relationship between PCs while controlling for host sex, age group, village, and schistosome infection intensity.

RESULTS

Parasite infection levels

The children came from 2 villages endemic for *S. haematobium* infection; an initial survey of 1980 permanent residents of the study villages aged 1–80 years showed that schistosome infection prevalence was significantly higher in Magaya (prevalence = 69%, 95% CI: 63% to 75%) than in Chitate (prevalence = 14%, 95% CI: 11% to 18%) ($\chi^2 = 187$, df = 1, $P < 0.001$), as was infection intensity 58 eggs/10 ml urine (Standard error of the mean (SEM) = 8·02) and 15 eggs/10 ml urine (SEM = 4·17) respectively ($F_{1,612} = 201$, $P < 0.001$). The World Health Organisation's classification guidelines classify Magaya as a high infection area (i.e. more than 50% of the population infected) and Chitate as a low infection area (i.e. less than 10% of the population infected) (WHO, 2002). Amongst the 1–5 year olds participating in this study, the overall prevalence of *S. haematobium* infection was 14·7%, (95% CI = 13–36·4%) with 13 out of 54 children in Magaya being infected and only 1 child from Chitate being infected (prevalence = 2·6%, 95% CI = 0·06%–13·5%). Infection prevalence was significantly different in the two villages ($X^2 = 7·8$, $P < 0.001$). Similarly infection intensity was significantly different in the two villages ($F_{1,94} = 5·88$, $P = 0.020$) with mean infection intensity in Magaya being 17·5 eggs per 10 ml urine (SEM = 9·5) and infection rage 0–458 eggs/10 ml urine and the only infected child in Chitate excreting 1 egg/10 ml urine. While the presence of schistosome infection was apparent from the youngest age group in Magaya, the high infection area (age of youngest egg positive child: 3 years 0 months), the only child excreting schistosome eggs in Chitate, the low

infection area, was aged 4 years and 6 months. Schistosome infection levels in Magaya and Chitate partitioned by age are shown in Table 1. These infection patterns were consistent when anti-egg IgM responses were used for diagnosing schistosome infection, which gave a prevalence of 24·4% in Magaya (95% CI = 12·9–39·5%) (the youngest infected person here was 3 years of age) and 7·7% in Chitate (95% CI = 1·6–20·9%) (the youngest infected person here was 4 years of age). The age profiles of schistosome infection measured by egg counts was comparable to that measured by the presence of egg-specific immune responses in both villages as shown in Fig. 1. There were no *P. falciparum* parasites detected in the blood of any of the children by thick or thin smears and these results were confirmed by the rapid Paracheck serological test.

Parasite-specific antibody responses

Schistosome–specific responses directed against cercariae and eggs were significantly higher than those directed against adult worms (Table 2). When compared to Zimbabweans, schistosome- and *Plasmodium*-naïve European controls had lower means and ranges of schistosome-specific antibodies as shown in Fig. 1A. When using these controls as cut-offs (i.e. mean ± 2 standard deviations), 43·4% of the children had levels of anti-cercariae IgM above those of European donors, indicating that these children had been exposed to cercariae antigens. 27·4% of the children (all of whom had anti-cercariae IgM levels above those of the controls) had anti-adult worm responses, above those of the controls. Thus significantly more children had been exposed to schistosome antigens than the proportion who were excreting schistosome eggs ($X^2 = 9·07$, $P = 0.005$). Furthermore, the proportion of children with anti-schistosome IgM levels (against all life stages) above that of the controls was significantly higher in Magaya (60%) compared to Chitate (23·7%, $X^2 = 10·7$, $P = 0.001$). In both villages, the proportion
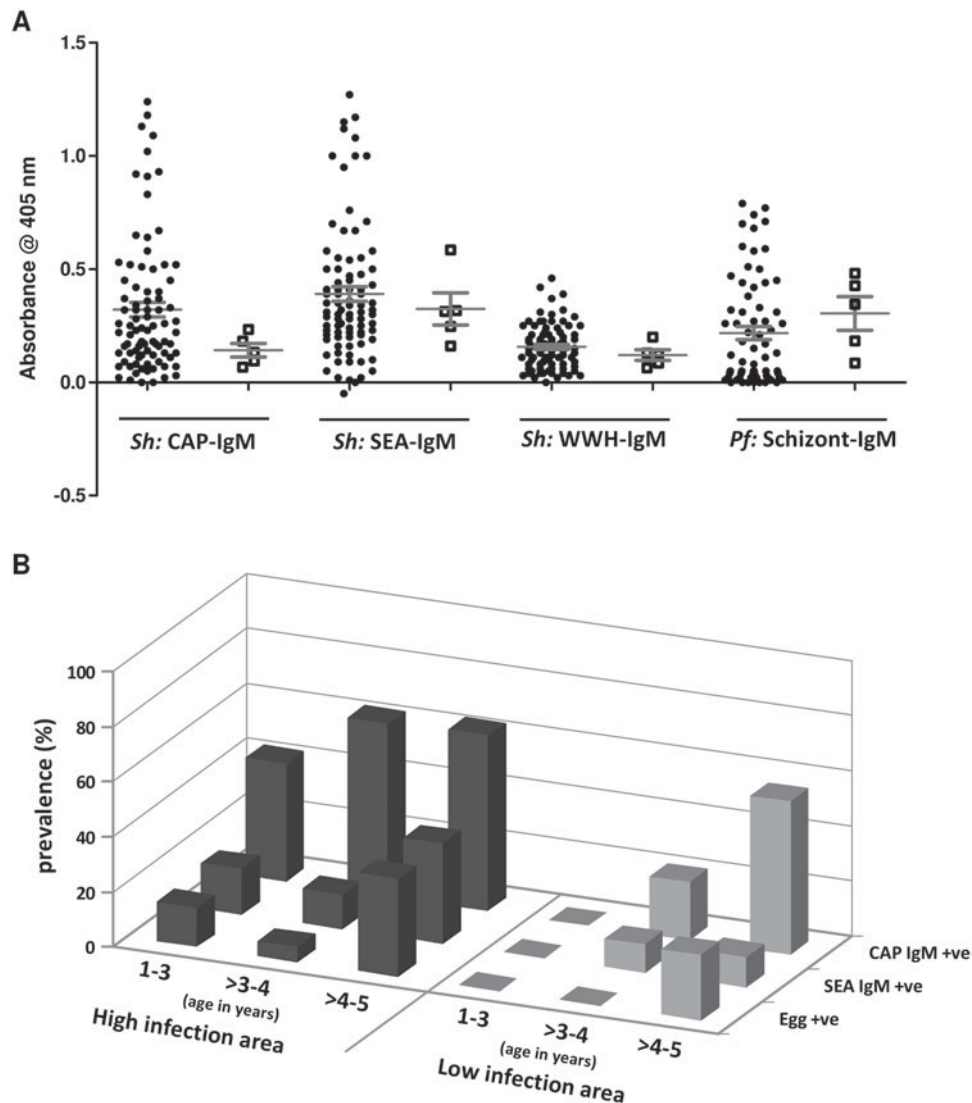
Fig. 1. Parasite-specific IgM responses. (A) Comparison of individuals' parasite-specific IgM antibody levels in the Zimbabwean study group (closed circles) compared to European donors (open squares) for each parasite antigen for *Schistosoma haematobium* (Sh) (CAP = cercariae antigen preparation, SEA = soluble egg antigen, WWH = soluble adult worm antigen) and *Plasmodium falciparum* (Pf) (Scz = schizont). Mean and standard error of the mean is shown for each population. (B) Comparison of schistosome infection prevalence determined by egg count and by egg-specific antibodies vs. presence and exposure to parasite antigens. The figure shows the percentage of people with at least 1 egg in 10 ml urine (Egg + ve), the percentage of people with anti-egg IgM levels (SEA IgM + ve) above the control cut-off and, the percentage of people with anti-cercariae (CAP IgM + ve) above the control cut-off taken to indicate exposure to cercariae.

of children showing evidence of exposure to schistosomes rose with age although this was not statistically significant (Fig. 1B). Titres of *Plasmodium* crude schizont-specific IgM were significantly higher than those for IgG (Z = − 5, 53, P <0·001), whilst among the recombinant antigens; titres were highest against MSP-2(Dd2) antigen. 13% of the children had anti-schizont IgM levels above those of the negative controls whose raw data (antibody titres) are shown in Fig. 1A.

Further analyses to determine the factors associated with antibody levels showed that there were no significant differences in antibody levels in male compared to female children. Levels of IgM directed against all schistosome parasite stages rose with host age (Fig. 2A) regardless of their village of residence; however this relationship was significant only for anti-worm IgM (Table 2). Age also had a significant effect on anti-cercariae IgG4. Anti-MSP-2(Dd2) rose with age (Fig. 2B) although this relationship was not significant (Table 2) while both IgM directed against *Plasmodium* total schizont and total IgG directed against MSP-2(CH150) declined with host age (Fig. 2B, Table 2). There were differences in the levels of parasite-specific responses mounted by children resident in the different villages. The majority of schistosome-specific responses were higher in Magaya (high infection area) compared to Chitate

Table 2. Analysis of variance F and (P) values of the factors affecting parasite-specific antibody levels and systemic cytokines

	Antibody/ Cytokine	Antigen	Sex	Age-group	Village	Infection status	Village*Age group interaction
S. haematobium	IgM	CAP	0·35(0·70)	0·87(0·42)	0·37(0·54)	1·27(0·26)	1·42(0·25)
		SEA	0·11(0·89)	0·43(0·65)	0·28(0·60)	2·36(0·13)	0·06(0·94)
		WWH	1·17(0·32)	**5·17(0·009)**	**15·37(<0·001)**	1·46(0·23)	0·19(0·83)
	IgE	CAP	0·72(0·49)	1·77(0·18)	**13·25(0·001)**	0·97(0·33)	0·56(0·57)
		SEA	0·12(0·89)	1·37(0·26)	1·70(0·20)	0·39(0·54)	**3·27(0·05)**
		WWH	0·15(0·87)	0·004(0·996)	2·30(0·14)	0·67(0·42)	1·11(0·34)
	IgG4	CAP	1·46(0·21)	**3·18(0·05)**	0·96(0·33)	1·74(0·19)	0·42(0·66)
		SEA	1·99(0·15)	1·08(0·35)	2·41(0·13)	8·52(0·005)	**3·08(0·05)**
		WWH	0·01(0·99)	0·96(0·39)	**6·59(0·013)**	3·40(0·07)	1·05(0·36)
P. falciparum	IgM	TSz	0·95(0·31)	**4·28(0·018)**	**66·56(<0·001)**	2·86(0·10)	0·42(0·66)
	IgG	TSz	0·80(0·45)	1·56(0·21)	**29·87(<0·001)**	1·85(0·18)	0·55(0·58)
		MSP-1$_{19}$	0·90(0·41)	0·83(0·43)	0·19(0·67)	0·11(0·74)	0·77(0·47)
		MSP-2 (CH150)	0·90(0·41)	14·21(<0·001)	**76·64(<0·001)**	0·34(0·56)	0·83(0·44)
		MSP-2(dD2)	0·45(0·62)	0·03(1)	1·67(0·20)	**4·49(0·038)**	0·79(0·46)
Cytokines	IFN-γ		0·22(0·64)	0·87(0·42)	0·04(0·84)	1·71(0·20)	0·06(0·95)
	TNF-α		1·38(0·25)	1·25(0·29)	0·17(0·68)	0·23(0·63)	0·35(0·71)
	IL-4		3·97(0·06)	2·29(0·11)	23·3(<0·001)	0·86(0·36)	**5·04(0·01)**
	IL-5		0·1(0·96)	0·15(0·86)	0·02(0·89)	1·51(0·22)	0·11(0·90)
	IL-10		**5·23(0·026)**	2·2(0·12)	0·23(0·63)	0·91(0·34)	0·78(0·46)

Schistosome-specific antibody levels were measured against cercariae (CAP), adult worm (WWH) and egg (SEA) antigens. *Plasmodium* antigens were measured against total schizont (Tsz) and the recombinant proteins Merozoite Surface protein (MSP)-1$_{19}$, MSP-2(CH150) and MSP-2(dD2). *P* values significant at $P < 0.05$ are highlighted in bold.

(low infection area) (Table 2). Only anti-worm IgG4 responses were higher in Chitate compared to Magaya. The converse was true for anti-*Plasmodium* responses, with the difference being significant only for responses against the schizont and MSP-2 (CH150). Having allowed for the effects of sex, age and village of residence, the relationship between infection intensity and antibody levels was tested. This showed that levels of all anti-schistosome antibodies were higher in egg positive children compared to egg negative children and the converse was true for *Plasmodium*-specific responses (Table 2). The relationship was significant only for anti-schistosome egg IgG4 and anti-*Plasmodium* MSP-2(Dd2). The interaction between village and age group had a significant effect on anti-schistosome egg IgE and IgG4, showing that the development of these responses with host age differ between the two villages.

Relationship between exposure and acquired immunity

Factor analysis reduced, all of the 9 schistosome-specific immune responses to 3 components shown in Table 3. The first component *Sh*-PC1 accounting for the highest variance was composed of all the anti-schistosome IgM responses and anti-egg IgE and IgG4. This component reflected exposure to schistosome antigens and was significantly associated with schistosome infection intensity, being higher in egg

positive children after allowing for host sex, age and village (Table 4A). The second component *Sh*-PC2 was composed of IgE directed against cercariae, previously shown to be associated with hypersensitivity in the skin. The only factor affecting levels of this component was village, with people in the low infection area having significantly higher levels than people residing in the high infection area. However, this component was significantly correlated with levels of *Sh*-PC1 after controlling for age, sex, village, or schistosome infection (Table 4B). The last component *Sh*-PC3 was made up of anti-worm IgE and IgG4. Anti-worm IgE was negatively loaded in component 3, implying an inverse relationship with anti-worm IgG4 (Table 4A). Thus low scores for this component are analogous to an increase in the adult worm-specific IgE:IgG4 ratio, which is associated with development of resistance to schistosome infection. *Sh*-PC3 was not significantly associated with host sex, age, village of residence or schistosome infection (Table 4A) but was positively correlated with the first component *Sh*-PC1 (Table 4B).

The five anti-*Plasmodium* antibody responses reduced to 2 principal components (Table 5), the first component, (*Pf*-PC1) explaining 42% of the variation was made up of the anti-schizont responses and anti MSP-2(Dd2) responses. These responses are indicative of exposure to *Plasmodium* schizont antigens and these responses declined significantly with
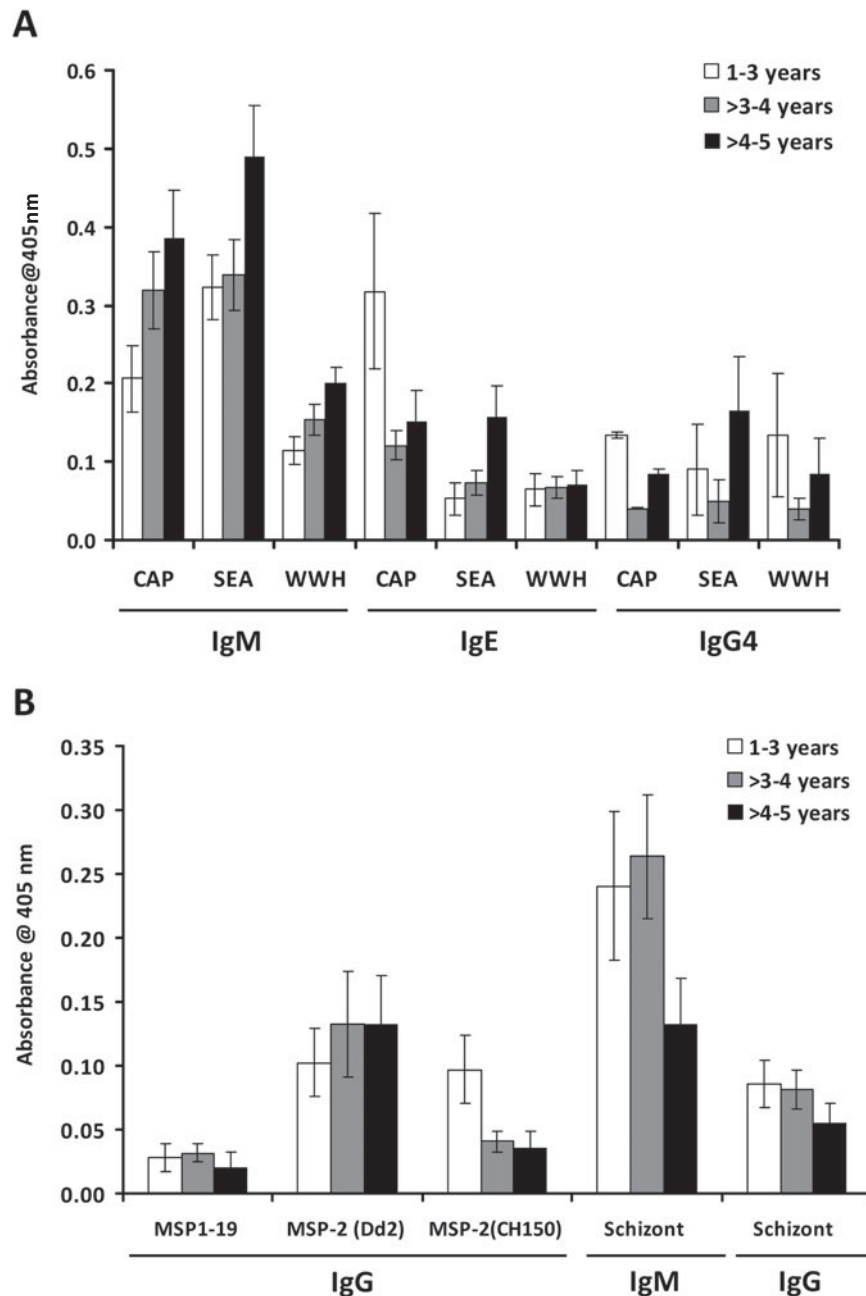
Fig. 2. Parasite specific age-antibody profiles. Open bars = 1–3 years old, grey bars = 3 > to 4 years, black bars = 4 > to 5 years. Standard error of the mean is shown for each bar. (A) *Schistosoma haematobium* -specific responses (CAP = cercariae antigen preparation, SEA = soluble egg antigen, WWH = soluble adult worm antigen preparation. (B) *Plasmodium falciparum*-specific responses (MSP = Merozoite Surface Protein).

age (Table 4A). Furthermore, these responses were significantly higher in Chitate, the low schistosome infection area but had no relationship to schistosome infection or host sex. Anti-MSP-1_{19} and MSP-2 (CH150) responses were grouped in component 2 (*Pf*-PC2), which explained 24% of the variation. These responses are indicative of the development of immunity against *Plasmodium* infection and were not related to host age, sex, village, or schistosome infection. However, they were negatively correlated to the *Pf*-PC1 responses after controlling for the effects of age, sex, village and schistosome infection (Table 4B).

Cytokine responses

To allow direct comparisons between cytokines, the number of people with detectable systemic cytokine levels was determined and compared rather than the absolute values (absolute levels of cytokines varied on different scales with IL-4 and IFN-γ levels being in ng/ml while levels of IL-10 were in pg/ml). Most participants had detectable levels of IL-10, followed by IL-4 with the least number of participants having detectable TNF-α (Fig. 3A). Only 2 children (both 5 years of age, one positive for schistosome eggs and the other negative) had detectable levels of all

Table 3. Principal components extracted for schistosome-specific responses

	S. haematobium component		
	PC1- Schistosome exposure (*Sh*-PC1)	PC2-Anti-schistosome egg and cercariae responses (*Sh*-PC2)	PC3-Adult-worm-specific immunity (*Sh*-PC3)
	Accounts for 36% of total variation	Accounts for 16% of total variation	Accounts for 12% of total variation
CAP IgM	**0·879**	−0·128	0·173
SEA IgM	**0·785**	−0·222	0·071
WWH IgM	**0·833**	−0·235	0·023
CAP IgE	0·355	**0·779**	0·008
SEA IgE	**0·646**	0·564	0·036
WWH IgE	0·101	0·409	**−0·728**
CAP IgG4	0·469	−0·328	−0·141
SEA IgG4	**0·637**	0·008	−0·019
WWH IgG4	0−0·138	0·367	**0·731**

Schistosome-specific antibody levels were measured against cercariae (CAP), adult worm (WWH) and egg (SEA) antigens. The loading value of each antibody for each principal component (PC) are given with variables with strong loadings (0·6 or $<-0·6$) are indicated in bold.

Table 4A. Analysis of variance F and (P) values of the factors affecting parasite exposure and immune responses

	Age-group	Village	Infection intensity	Village* age group
Sh-PC1 (exposure to schistosome infection)	0·9(0·41)	1·02(0·37)	3·25(0·08)	**10·04(0·002)**	0·67(0·52)
Sh-PC2 (cercariae-pecific IgE responses)	1·68(0·20)	1·98(0·15)	**13·57(0·001)**	0·01(0·922)	2·16(0·13)
Sh-PC3 (adult worm-specific IgE)	0·12(0·89)	0·30(0·74)	0·02(0·88)	0·31(0·58)	0·70(0·50)
Pf-PC1 (exposure *to Plasmodium infection*)	0·85(0·43)	**4·07(0·02)**	**56·0(P<0·001)**	1·05(0·31)	0·16(0·84)
Pf-PC2 (Immunity against *Plasmodium*)	0·85(0·43)	0·27(0·76)	0·53(0·47)	0·13(0·72)	1·02(0·37)
Cytokine –PC (systemic cytokines)	1·85(0·18)	1·13(0·33)	2·76(0·10)	**3·97(0·05)**	0·60(0·55)

Factors significant at $P<0·05$ are highlighted in bold.

Table 4B. Relationship between parasite-specific antibody levels and systemic cytokines

	Sh-PC1	*Sh*-PC2	*Sh*-PC3	*Pf*-PC1	*Pf*-PC2
Sh-PC1 (exposure to schistosome infection)	—				
Sh-PC2 (cercariae-specific IgE responses)	**0·33 (0·02)**	—			
Sh-PC3 (adult worm-specific IgE)	0·01(0·315)	−0·22(0·08)	—		
Pf-PC1 (exposure *to Plasmodium infection*)	0·20(0·09)	−0·96(0·27)	−0·17(0·14)	—	
Pf-PC2 (Immunity against *Plasmodium*)	−0·191(0·11)	0·03(0·42)	0·19(0·11)	**−0·34(0·01)**	—
Cytokine –PC (systemic cytokines)	0·21(0·08)	**0·28(0·04)**	−0·17(0·13)	−0·03(0·42)	−0·04 (0·39)

Partial correlation (controlling for host sex, age, village and schistosome infection intensity) r-values and (P) values. Correlations significant at $P<0·05$ are highlighted in bold.

5 cytokines. The proportion of children producing each cytokine did not vary significantly with host age, sex, and village or schistosome infection. When the effects of host sex, age, village and schistosome infection on the concentrations of each cytokine was assessed, sex was found to significantly affect levels of IL-10, with females having higher levels than males.

The relationship between IL-4 and age was also found to differ between the two villages (Table 2).

Factor analysis reduced all the cytokines into a single variable (Table 6) composed of IL-5, IL-10 and IFN-γ. Levels of this cytokine PC rose significantly with age and were positively correlated with schistosome infection (Table 4A) and the

Table 5. Principal components extracted for *Plasmodium*-specific responses

	P. falciparum component	
	PC1-Exposure to *Plasmodium falciparum* (*Pf*-PC1)	PC2-Immunity against *Plasmodium falciparum* (*Pf*-PC2)
	Accounts for 42% of total variation	Accounts for 24% of total variation
MSP-1$_{19}$ IgG	0·263	**− 0·667**
MSP-2(Dd2) IgG	0·049	**0·735**
MSP-2(CH150) IgG	**0·627**	0·445
Tsz IgM	**0·903**	− 0·110
Tsz IgG	**0·900**	− 0·045

Plasmodium antigens were measured against total schizont (Tsz) and the recombinant proteins Merozoite Surface protein (MSP)-1$_{19}$, MSP-2(CH150) and MSP-2(dD2). The loading value of each antibody for each principal component (PC) are given with variables with strong loadings (0·6 or − 0·6) are indicated in bold.
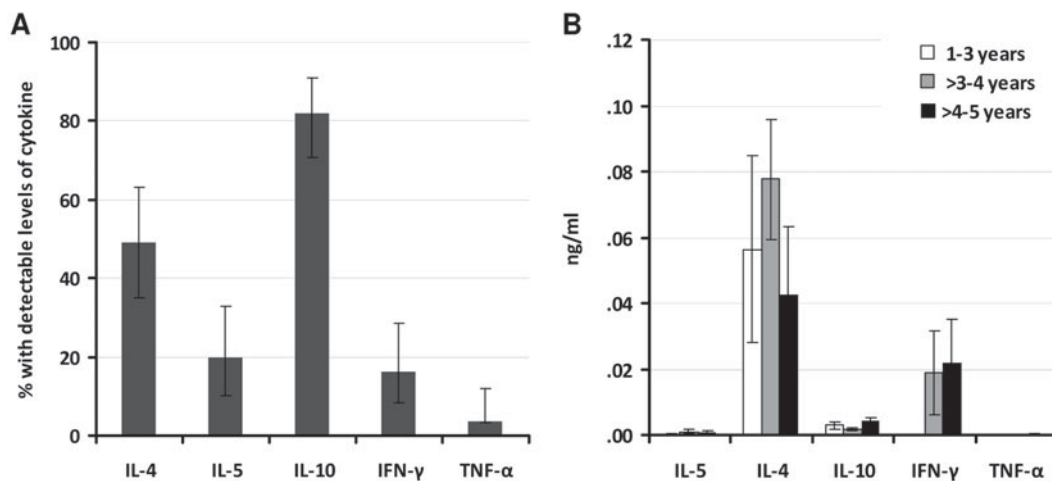

Fig. 3. Systemic levels of the cytokines. (A) Percentage of people with detectable cytokine levels in their plasma with 95% confidence interval shown. (B) Age-profile for each cytokine. Open bars = 1–3 years old, grey bars = 3 > to 4 years, black bars = 4 > to 5 years. Standard error of the mean is shown for each bar.

schistosome-specific hypersensitivity responses in *Sh*-PC2 (Table 4B) after controlling for host age, sex, village or schistosome infection.

DISCUSSION

The geographical and demographical co-distribution of *Schistosoma haematobium* and *Plasmodium falciparum* in Africa means that young children can be co-infected with both parasites. Despite this overlap, the burden of these co-infections in children aged 5 years and under is relatively less studied than in children aged 6 years and above. Furthermore, there is a paucity of studies characterizing the nature and development of parasite-specific immune responses to these parasites in children aged 5 years and below. Therefore we set out to (1) determine the prevalence and intensity of infection and co-infection with these

parasites in children aged 1–5 years resident in a schistosome and *Plasmodium* endemic area; (2) determine levels of exposure detected as the presence of serum antibodies specific for one or both parasites and, (3) characterize systemic immune responses (cytokine and parasite-specific antibody) in these young children. By conducting the study in two villages of different schistosome infection levels, the effect of different schistosome transmission dynamics on immune responses could be compared.

In this study, the overall schistosome infection prevalence was 14·7% by egg count, with a significant difference between the two areas. Prevalence in the high infection area was 23% and 3% in low infection area. These levels were slightly lower than those diagnosed by the presence of anti-egg antibodies (16·7% overall and 24·4% in Magaya and 7·7% in Chitate), consistent with a recent report from a study in

Table 6. Principal components extracted for
systemic cytokine levels

	Cytokine PC
	Accounts for 40% of total variation
IL-4	− 0·377
IL-5	**0·780**
IL-10	**0·674**
IFN-γ	**0·652**
TNF-α	0·587

The loading value of each antibody for each principal
component (PC) are given with variables with strong
loadings (0·6 or − 0·6) are indicated in bold.

pre-school Ugandan children exposed to *S. mansoni*
(Stothard *et al*. 2011) which showed that serological
diagnosis gave slightly higher but not significantly
different infection levels . In our study all egg-positive
children were also serologically positive for schisto-
some infection although there were some egg-negative
children who were positive for anti-egg antibodies.
Not surprisingly, more children showed evidence of
exposure to the infective stage of schistosomes,
cercariae, with 43·4% of the children producing anti-
cercariae IgM (60% in the high infection area and
23·7% in the low infection area) than those that were
positive for infection. The anti-cercariae responses
indicated in part, pre-patent, single sex or low
intensity infections which are not easily diagnosed
by egg count methods (Stothard *et al*. 2011).

The youngest children showing evidence of
exposure to cercariae antibodies were 3 years and
4 years in the high schistosome infection and low
schistosome infection areas, respectively. This differ-
ence was consistent with the diagnosis of schistosome
infection via microscopic egg counts with the young-
est age at which children started excreting schisto-
some eggs in the urine, 3 years and 4 years and
6 months in the two areas, respectively. We have pre-
viously reported that, in areas of high *S. haematobium*
infection, children as young as 4 months showed
immunological evidence of exposure to schistosome
cercariae (Woolhouse *et al*. 2000). Our current study
indicates that, in areas of high schistosome infection,
a large proportion of these young children are
exposed to schistosome cercariae and that this
exposure translates to patent infections in some cases.

The importance of these childhood schistosome
infections is augmented by their potential interaction
with other co-infections such as *Plasmodium*. Several
studies have reported that co-infected children suffer
parasite-related morbidity exacerbated by the pres-
ence of the other parasite (Remoue *et al*. 2003;
Arinola, 2005; Wilson *et al*. 2008; Diallo *et al*. 2010).
Conversely, there have been reports of a negative
association between helminth infections and the

severity of morbidity arising from *Plasmodium*
infections (Nacher *et al*. 2000; Lyke *et al*. 2005).
Experimental and human observational studies
have indicated that the exacerbation of morbidity as
well as negative association between helminth and
Plasmodium infections has an immunological basis
(Remoue *et al*. 2003; Arinola, 2005; Wilson *et al*.
2008; Diallo *et al*. 2010). In this current study, none
of the children were positive for *Plasmodium* infection
but 13·4% of the children showed evidence of recent
exposure to *Plasmodium* schizonts. It is unlikely that
this IgM response was related to maternal exposure to
Plasmodium parasites since the youngest child with
anti-schizont levels above those of the controls was
4 years old. All children showing immunological
evidence of exposure to the schizonts were resident in
the low schistosome infection area; the reasons for
this are unclear. Both study areas are mesoendemic
for *Plasmodium* infection but the differences in
temporal patterns of the rivers in the two areas that
affect the schistosome transmission dynamics may
affect the breeding sites for the *Plasmodium* mosquito
vector. There was no association between schisto-
some-specific and *Plasmodium*-specific immune re-
sponses in either village. This is possibly due to the
low level of *Plasmodium* endemicity in the area. Thus,
the interactions reported from the other studies
where both the *Plasmodium* transmission and the
incidence of clinical malaria is higher may be influ-
enced by current levels of parasitaemia experienced
by the children as well as their history of infection
with both parasites. We have previously reported an
interaction between schistosome-specific antibodies
and *P. falciparum* from older Zimbabwean children
resident in higher transmission areas for both
parasites (Mutapi *et al*. 2000, 2007*a*).

Immune responses against schistosomes and those
against *Plasmodium* parasites show distinct age
profiles (Mutapi *et al*. 1997; Mutapi *et al*. 2007*b*;
Iriemenam *et al*. 2009). In population studies in-
cluding children aged 6 years and above, schistosome
infection intensity, IgM and total IgG antibodies
follow the typical convex age-infection curve orig-
inally described for schistosome infections by Fisher
in 1934 (Fisher, 1934), i.e. they rise with age peaking
in older children and then decline in adulthood. This
age-profile for IgM and total IgG is largely a result of
age-related exposure patterns to infective water as
well as egg and adult worm antigens (Ndhlovu *et al*.
1996; Mutapi *et al*. 1997). Unlike IgM and total IgG,
levels of IgE levels with age (Dunne *et al*. 1992;
Mutapi *et al*. 1997) and the ratio of IgE:IgG4, which
has been associated with the development of resist-
ance to infection/re-infection in *S. haematobium*
infections, also increases with age. This study showed
that these age-related patterns can already be detected
in children under 6 years of age. IgM levels directed
against all parasite stages rose with age consistent
with age-related increases in exposure to infective

water (Woolhouse *et al.* 2000). Furthermore, IgM levels rose more quickly with age in the high infection area consistent with the existence of peak shifts in antibody levels in areas of different schistosome infections (Mutapi *et al.* 1997). Schistosome-specific antibody responses were higher in Magaya, the high schistosome infection area compared to Chitate the low infection area. This is not surprising as previously studies have indicated that in the early age groups schistosome specific responses are correlated to the levels of the infections that are stimulating them (Ndhlovu and Woolhouse, 1996; Mutapi *et al.* 1997). IgE responses directed against cercariae were significantly higher in the low infection area compared to the high infection area and they declined with age (although this was not significant) which would be consistent with a reduction in acute immune hyperactivity to cercarial exposure (swimmers' itch) (Cort, 1928; Kourilova *et al.* 2004) mediated by IgE antibodies which is observed in residents of schistosome-endemic areas. This suggests that by the time these children start school (the youngest age-group normally enrolled in schistosome immuno-epidemiology studies) the early anti-cercariae responses are already different from those observed at first exposure. Thus in this study the immune responses which are associated with exposure to the parasite antigens are significantly related to schistosome infection. However, immune responses associated with protection to adult schistosomes did not follow distinct patterns with infection. Similar to *Plasmodium* infections, acquired immunity to schistosomes takes several years to develop to levels sufficient to have observed effects on levels of infection/re-infection (Langhorne *et al.* 2008; Woolhouse *et al.* 1991).

The *Plasmodium* antibody responses in these children were reduced into two variables composed of responses directed against the crude antigens and MSP-2(CH150) and those directed against MSP-1$_{19}$ and MSP-2(Dd2) suggesting that these variables were representative of distinct biological processes associated with putative exposure to parasites (Naus *et al.* 2003*b*) and the development of protective acquired immunity respectively (Conway *et al.* 2000; Mawili-Mboumba *et al.* 2003; Metzger *et al.* 2003; Polley *et al.* 2003, 2006; Cavanagh *et al.* 2004). These responses were negatively correlated and *Plasmodium*-specific responses indicative of exposure to parasite antigens declining significantly with age (after controlling for variation to host sex, village, and schistosome infection). Unlike anti-schistosome antibody responses, levels of antibodies to several *Plasmodium* antigens may vary with the seasonality of parasite transmission, often being higher during periods of high transmission than at the end of a low transmission season (Cavanagh *et al.* 1998). Furthermore, levels of anti-*Plasmodium* antibodies tend to be higher in individuals carrying patent parasite infections than in those without parasites at the time of the survey for both crude antigens and recombinant antigens such as the vaccine candidates we studied here (Polley *et al.* 2006; Osier *et al.* 2008). Isotype switching after B cell activation and the absence of IgM memory responses also means that levels of circulating anti-*Plasmodium* IgM responses decline in favour of a switch to IgG3 responses typically observed in children (Taylor *et al.* 1998). Therefore it is not surprising that immune responses indicative of recent exposure decline with age and responses associated with resistance to *Plasmodium* did not show distinct patterns with age as these are still developing.

Systemic cytokines were detected in most children, with IL-10 being the most prevalent within the cohort, despite being present at low concentrations compared to the other cytokines. This is consistent with results we have previously reported from Zimbabwean populations showing that levels of both schistosome-specific and systemic IL-10 were low compared to the other cytokines (Mutapi *et al.* 2007*b*; Milner *et al.* 2010). The high prevalence of IL-10 may be related to its role in isotype switching to IgG subclasses (Garraud *et al.* 2003) and its regulatory function modulating both Th1 and Th2 responses (Hunt and Grau, 2003). The least detected cytokines were TNF-*α* and IFN-*γ*, which are associated with inflammatory Th1-type responses. The cytokine responses rose with schistosome specific anti-egg responses and schistosome infection. We have previously demonstrated that systemic IL-5 and IL-4 were correlated with low *S. haematobium* infection in putatively resistant individuals (i.e. lifelong residents of schistosome-endemic areas who, despite continued exposure to infective water, harbour little or no schistosome infection) (Milner *et al.* 2010). In human *Plasmodium* infections elevated levels of both IFN-*γ* and TNF-*α* are associated with severe malaria (Awandare *et al.* 2006) so that their presence in children concurrently exposed to helminth and *Plasmodium* infections might be expected to exacerbate *Plasmodium*-related pathology. Conversely, low IL-10 plasma levels are associated with severe malarial anemia (Kurtzhals *et al.* 1998). IL-5 and IL-10 are associated with protection against re-infection with schistosomes (Medhat *et al.* 1998; Abath *et al.* 2006). However, there were no significant associations between plasma cytokines and *Plasmodium*-specific antibody levels. This suggests that either the plasma cytokines reflect current parasitic infections which is consistent with the significant association between the systemic cytokine levels and schistosome infection, or, that the dynamics of parasite-specific and systemic cytokine differ in this context.

Overall this study has shown that, in both areas of high and low schistosome infection, children aged 5 years and below show immunological evidence of exposure to schistosome infection. In areas of high

schistosome infection, a large proportion of children aged 5 years and below was excreting schistosome eggs. Furthermore, the children also show immunological evidence of exposure to *Plasmodium* although none of them were positive for *Plasmodium* infection. Both schistosome-specific and *Plasmodium*-specific responses associated with protection to infection/re-infection were detected in the children together with a mixed systemic cytokine phenotype consisting of both Th1 and Th2 cytokines. The immune responses measured showed age-related patterns consistent with those reported in older children, suggesting that the development of protective acquired immunity starts in early childhood, although the effects of this immunity on infection levels and pathology may take time to become apparent.

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