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Human cytokine responses during natural and experimental exposure to parasitic helminth infection

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Abstract

Over one third of the human population is currently infected by one or more species of parasitic helminth, but the immune responses elicited by these infections remain poorly defined. Studies in helminth-exposed human populations and laboratory models suggest that helminth infection elicits a range of different effector cell types and that protective immunity and resistance to immune-mediated pathology depends on the balance between these responses. The aim of this thesis was to investigate how cytokines, the molecular mediators of the immune system, can be used to characterise human immune phenotype during natural and experimental helminth infection. Cytokines associated with innate inflammatory (TNFα, IL-6 and IL-8), Th1 (IFNγ, IL-2 and IL-12p70), Th2 (IL-4, IL-5 and IL-13), Th17 (IL-17A, IL-21 and IL-23) and regulatory (IL-10 and TGFβ) immune phenotypes were analysed to provide the most comprehensive analysis of cytokine responses in human helminth infection conducted to-date. Using a multivariate statistical approach cytokines were analysed as combined immune profiles to reflect their complex interactions *in vivo*.

In the first part of the study venous blood samples collected from a cross-sectional cohort of 284 Zimbabweans (age range: 3 -86 years) endemically-exposed to *Schistosoma haematobium* were cultured with antigens from different stages of the parasite's life-cycle (cercariae, adult worms and eggs) and the anti-schistosome vaccine candidate antigen glutathionine-S-transferase (GST). Cytokines responses were quantified in culture supernatants via enzyme-linked immunosorbent assay (ELISA). These assays were repeated 6 weeks after clearance of infection by anti-helminthic treatment. Parasitological and demographic characterisation of the cohort before, 6 weeks, 6 and 18 months after treatment allowed cytokine responses to be related to epidemiological patterns of infection before treatment and the risk of re-infection after treatment. The main findings of this study were:

- Cytokine responses to the antigens of *S. haematobium* cercariae are more pro-inflammatory than those elicited by adult worms and eggs prior to treatment, reflecting the distinct proteomes and exposure patterns of the 3 life-cycle stages
- Young children (5-10 years old) have a more regulatory and Th17-polarised cytokine response to *S. haematobium* antigens than older children and adults. These responses are significantly associated with schistosome infection intensity and may contribute to the development of resistance to schistosomiasis with age and exposure to infection
- Anti-helminthic treatment leads to a shift in *S. haematobium* cercariae, egg and GST-specific cytokine responses towards a more pro-inflammatory phenotype
- The magnitude of change in *S. haematobium*-specific cytokine profiles after treatment is dependent on schistosome infection intensity at the time of treatment
- Individuals who remain un-infected up to 18 months after treatment to clear schistosome infection have a more pro-inflammatory and IL-21-polarised response to *S. haematobium* antigens 6 weeks after treatment than those who become re-infected, suggesting that post-treatment cytokine profiles promote resistance to re-infection

The second part of the study assayed systemic, parasite and allergen-specific cytokine responses in 45 adults with seasonally exacerbated allergy to grass pollen who were experimentally exposed to *Trichuris suis*. Cytokine responses in infected individuals were compared to those of 44 un-infected controls. This aspect of the study showed that:

- Exposure to *T. suis* promotes systemic and parasite-specific Th2 and regulatory cytokine responses, but does not alter cytokine responses to environmental allergens

Declaration

I declare that this thesis is my own work and I composed all chapters and conducted all data collection, laboratory and statistical analysis unless otherwise stated. All work which I did not conduct personally is fully acknowledged in the text. This work has not been submitted for any other degree or professional qualification.

Claire Bourke
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Abbreviations

A – Chance-corrected within-group agreement

ADCC - Antibody-dependent cell-mediated cytotoxicity

ANOVA – Analysis of variance

APC – Antigen-presenting cell

B – Beta coefficient

BMDC - Bone marrow-derived dendritic cell

BSA – Bovine serum albumin

CAP – Cercarial antigen preparation

CBA – Cytometric bead array

CD – Cluster of differentiation

CI – Confidence interval

Δ – Change in…

δ - Weighted mean within-group distance

DAI – Disease activity index

DALYs - Disability-adjusted life years

DC – Dendritic cell

df – Degrees of freedom

ε – Epsilon lower bound-adjusted degrees of freedom

ELISA – Enzyme-linked immunosorbent assay

E/S - Excretory/secretory product

F – F statistic

FcεR – IgE receptor

Foxp3 – Fork-head box P3

g6 – (timothy) grass pollen allergen

GCP - Good Clinical Practice

GI – Gastrointestinal

GPS - Global positioning systems

GST - 28kDa glutathionine-S-transferase (Sh – *S. haematobium*, Sm – *S. mansoni*)

HIV - Human immunodeficiency virus

H-L – Hosmer-Lemeshow statistic

HRP – Horseradish peroxidase

IBD - Inflammatory bowel disease

Ig – Immunoglobulin

IL – Interleukin

IFNγ – Interferon gamma

inflamm. – Inflammatory

L3 - Infective-stage 3 free-living larvae

LNFPIII - Lacto-N-fucopentanose III

MANOVA – Multivariate analysis of variance

MBP – Maltose binding protein

MCRZ - Medical Research Council of Zimbabwe

MHC II – Major histocompatibility complex II

MLEC – Mink lung epithelial cell

MRI - Magnetic resonance imaging

mRNA – Messenger ribonucleic acid

MRPP – Multiple response permutation procedure

MS – Multiple sclerosis

NIH – National Institute of Health

NLR – NOD-like receptors

NMS – Non-metric multidimensional scaling

NS or ns – Not significant

p – p-value

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PC – Principal component

PE – Phycoerythrin

PGD_2 - Prostaglandin D_2

PHA – Phytohaemagluttanin

PMA - Phorbol myristate acetate

PRR - Pathogen recognition receptor

r – Pearson's r

r^2 – Pearson's r-squared

RA - Radiation-attenuated

SAP – Schistosomula antigen preparation

SEA – Soluble egg antigen

Sm97 – *S. mansoni* paramyosin

spp. – Species

STAT – Signal transducer and activator of transcription

STH - Soil-transmitted helminths

T – Test statistic

t3 – Birch pollen allergen

T eff - CD4+ T (helper) effector cell

TGFβ – Transforming growth factor beta

Th – CD4+ T helper cell

TLR - Toll-like receptor

TMB - 3,3,5,5-tetramethylbenzidine HRP substrate solution

TNFα – Tumour necrosis factor alpha

TPI – Triose phosphate isomerase

Teff – CD4+ T (helper) effector cell

Treg – CD4+ T (helper) regulatory cell

TSO – *Trichuris suis* ova

UZ - University of Zimbabwe

UZIRB - Institute Review Board of the University of Zimbabwe

W X^2 – Wald chi-squared statistic

W.H.O. – World Health Organisation

WWH – Whole (adult) worm homogenate

+ve – Positive

-ve – Negative

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Chapter 1

General Introduction

Parasitic helminths are evolutionarily ancient human pathogens (Cox 2002) and the causative agents of highly prevalent and debilitating diseases world-wide (Hotez *et al.* 2008). Despite their high prevalence there remains no effective vaccine against these infections and less than 5% of those at risk of infection have access to anti-helminthic treatment (Gryseels *et al.* 2006; Hotez 2009). One of the major obstacles to eradicating human helminthiases and the morbidity they cause is that the immune responses that these parasites elicit in humans are ill-defined, particularly in endemically-exposed populations where hosts are repeatedly exposed to infection throughout their lives. In this thesis I investigate how immune responses to helminths can be better characterised in humans, focusing particularly on cytokine responses which are the molecular mediators of the immune response.

Schistosoma spp. worms are amongst the most significant helminth species affecting global health and over 650 million people are at risk of infection (Chitsulo *et al.* 2000). In chapters 3-6 I investigate how the schistosome-specific cytokine response is influenced by differences in the parasite life-cycle and epidemiological patterns of infection in a schistosome-endemic population and whether these responses are related to the development of immune-mediated resistance before and after anti-helminthic treatment. This chapter provides an introduction to the immunobiology and epidemiology of schistosomiasis and discusses the paradigms from mathematical and laboratory models of infection that are yet to be tested in human population studies.

One of the major features of parasitic helminths is their ability to modulate the host immune response (Maizels *et al.* 1993; Maizels and Yazdanbakhsh 2003; Maizels *et al.* 2004). Whilst this feature may contribute to their persistence during natural infections, recent interest has developed into whether this feature could be exploited by using experimental helminth infections to regulate immune-mediated diseases (Reddy and Fried 2007; Erb 2009). The latter part of this chapter will provide an overview of the clinical trials of 'helminth therapy'

conducted to-date and how these studies have contributed to our understanding of human immune responses to helminth infections in previously un-exposed hosts.

1.1 Parasitic helminth infections

The most prevalent human helminthiases are caused by nematode species including filarial worms (*Brugia malayi*, *Onchocerca volvulus*, and *Wuchereria bancrofti*) and soil-transmitted helminths (STH): *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm (*Ancyclostoma duodenale* and *Necator americanus*), *Strongyloides* spp. and *Enterobius vermicularis* (Hotez *et al.* 2008). Of the trematodes, *Schistosoma* spp. are the most widely distributed and cause the greatest burden to public health (Gryseels *et al.* 2006). There are also many important human cestode parasites, often called 'tapeworms' (Zhang *et al.* 2008).

Although parasitic helminths have a diverse phylogeny, they share a number of features that have contributed to their success as parasites of humans. Firstly, they are large, multicellular organisms ranging from millimetres to metres in length (Anderson and May 1992) and thus the immune response directed against them differs markedly to that elicited by micro-parasites (e.g. bacteria and viruses), which can be readily internalised by immune cells. Parasitic helminths also tend to have complex life-cycles made up of several distinct stages highly specialised to infect, inhabit and exit their human hosts respectively. Variation between the life cycles of different helminth species, summarised in Table 1.1, reflects the selective challenges posed by the different geographical environments and intra-host niches that they exploit (Bourke *et al.* 2011) and is discussed in more detail for the human schistosomes later in this chapter. This adaptation is also evident at a molecular level since the large genomes of parasitic helminths correspond to a diverse range of expressed proteins with which the host immune response can interact (Ghedin *et al.* 2007; Berriman *et al.* 2009; Zhou *et al.* 2009; Mitreva *et al.* 2011). Identification of helminth homologues of human proteins (Pastrana *et al.* 1998; Gomez-Escobar *et al.* 2000) and rapid evolution among parasite secreted proteins (Hoekstra *et al.* 2000; Harcus *et al.* 2004) suggest that helminth antigens exposed to (and interacting with) the host immune system have diversified in response to the selective pressures associated with parasitism. Thus helminths are evolutionarily adapted for long-term interactions with their hosts and tend to establish chronic infections. The average life-span of mature parasites in their human host ranges from 1-10 years (see Table 1), although in rare cases individual parasites have been reported to survive for up to 30 years (Christopherson 1924; Berberian *et al.* 1953; Harris *et al.* 1984).

	Gastrointestinal (GI) nematodes					Filarial nematodes			*Schistosoma* spp. trematodes		
	A. lum	***E. ver***	***N. ame***	***Stro. spp.***	***T. tri***	***B. mal***	***O. vol***	***W. ban***	***S. hae***	***S. jap***	***S. man***
Distribution	Af, As, L.Am	-	Af, As, L.Am	Af, As, L.Am	Af, As, L.Am	S.E.As	SS.Af, L.Am	As, SS.Af, L.Am	SS.Af	China, S.E.As	SS.Af, Brazil
Human infections (millions)	807	-	576*	30-100**	604	-	37	120	207***	207***	207***
Intermediate host	None	None	None	None	None	*Ano* spp.	*Sim* spp.	*Ano, Cul, Aed* spp.	*Bul* spp.	*Onc* spp.	*Bio.* spp.
Transmission route	Faeco-oral	Faeco-oral	Percutaneous	Percutaneous	Faeco-oral	Vector	Vector	Vector	Percutaneous	Percutaneous	Percutaneous
Maturation delay	50-80 days	15-43 days	40-50 days	-	50-84 days	-	365 days	-	21-28 days	25-30 days	25-30 days
Adult worm life-span	1-2 years	<1 year	2-3 years	-	1-2 years	-	8-10 years	3-5 years	3-5 years	3-5 years	6-11 years^
Adult worm length	15-35cm	2-13mm	7-11mm	-	~4cm	13-55mm	19-50mm	40-100mm	7-20mm	7-20mm	7-20mm
Adult worm niche	Ileum	Caecum	Ileum	Caecum	Caecum	Lymph	Skin	Lymph	Blood (bladder)	Blood (gut)	Blood (gut)
Fecundity per female	200,000 eggs/day	-	3000-6000 eggs/day	-	50-84 eggs/day	-	1000-2000 mf/day	-	3000 eggs/day	100-300 eggs/day	100-300 eggs/day

Table 1.1. Summary of the distribution and life-cycles of the major helminth species affecting human health (data collated from (Anderson and May 1992; Maizels *et al.* 1993; Gryseels *et al.* 2006; Hotez *et al.* 2008). *all hookworm species, ***Strongyloides stercoralis* only, ***all *S. haematobium*, *S. japonicum* and *S. mansoni* infections, ^Life-span of *S. mansoni* estimated using maximum likelihood techniques to assess pre and post-treatment field data (Fulford *et al.* 1995). *A.lum – Ascaris lumbricoides, E.ver – Enterobis vermicularis, N.ame – Necator americanus, Stro spp. – Strongyloides spp., T.tri – Trichuris trichiura, B.mal – Brugia malayi,, O.vol – Onchocerca volvulus, W.ban – Wuchereria bancrofti, S.hae – S. haematobium, S.jap – S. japonicum, S.man – S. mansoni,* Af – Africa, As – Asia, L.Am – Latin America, S.E.As – South East Asia, SS.Af – Sub-Saharan Africa, *Ano* spp. – *Anopheles* spp. mosquito, *Aed* spp. – *Aedes* spp. mosquito, *Cul* spp. – *Culex* spp. mosquito, *Bio.* spp. – *Biomphalaria* spp. (aquatic snail), *Bul* spp. – *Bulinus* spp. (aquatic snail), mf – microfilariae

1.2 Human schistosomiasis

1.2.1 Global health burden

The 3 predominant *Schistosoma* species affecting humans are *S. haematobium*, *S. japonicum* and *S. mansoni* which collectively account for over 200 million current infections(Fenwick *et al.* 2003; W.H.O. 2006) and an estimated 15,000 deaths per year (Fenwick *et al.* 2003). Infections are almost exclusively aggregated in areas of rural poverty, particularly in sub-Saharan Africa (Platt and Brooks 1997; Gryseels *et al.* 2006) where co-infections with other helminth species, *Plasmodium* parasites and human immunodeficiency virus (HIV) are common (Raso *et al.* 2004; Brooker *et al.* 2006; Midzi *et al.* 2008b). *S. intercalatum* and *S. mekongi* also cause human infections, but have a more restricted distribution (Gryseels *et al.* 2006). The global distribution of human schistosome infections is summarised in Figure 1.1. In addition to the mortality that they cause, schistosome infections account for significant chronic morbidities in infected individuals quantified globally as between 6 and 13.5 million disability-adjusted life years (DALYs) per year (King *et al.* 2005). Unfortunately, the paucity of data on the number of cases, particularly in young children (Stothard *et al.* 2011), and the difficulty in identifying subtle and chronic morbidities due to infection (King 2007) mean that these figures are almost certainly an under-estimate

1.2.2 Life-cycle

Schistosomes are dioecious trematode worms that have evolved a complex life-cycle comprising 2 intra-host and 2 free-living stages, summarised in Figure 1.2. The global distribution of schistosome infections (see Figure 1.1) is determined by the distribution of intermediate snail host species in which schistosomes replicate asexually; aquatic *Bulinus* spp. and *Biomphalaria* spp. for *S. haematobium* and *S. mansoni* respectively and amphibious *Oncomelania* spp. for *S. japonicum* (Gryseels *et al.* 2006). The definitive human host is exposed to 4 distinct life cycle stages of the parasite-infective larvae (cercariae), migratory larvae (schistosomula), mature adult worms and transmission stage parasites (eggs).

Humans become infected via contact with fresh water containing cercariae released from patently infected snails. In schistosome-endemic regions infection usually occurs during

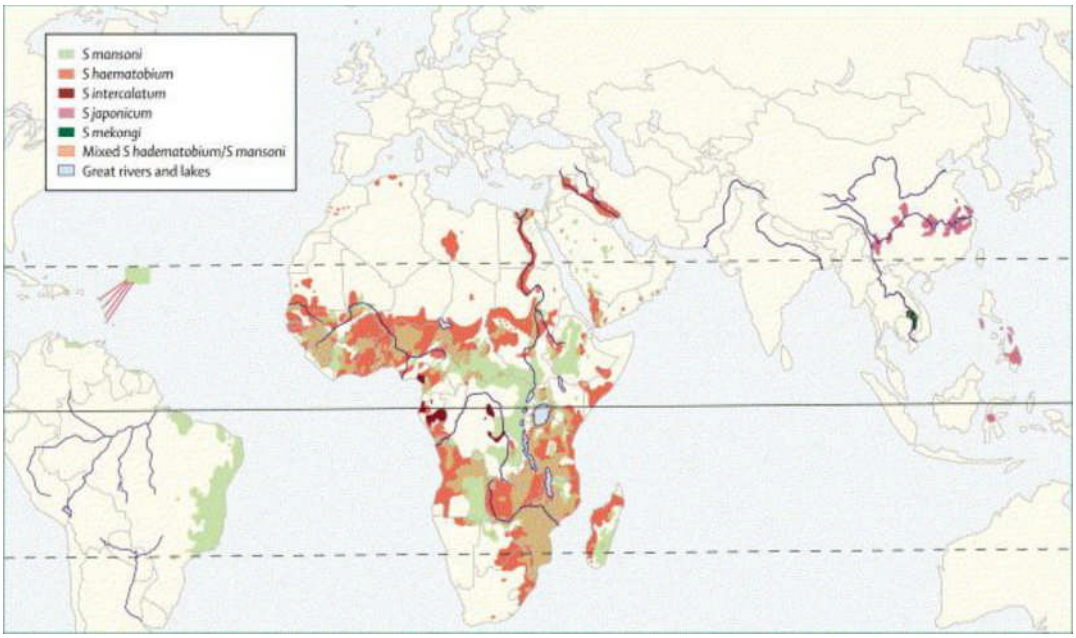

Figure 1.1. The global distribution of human schistosomiasis, reproduced from an article by Gryseels *et al*, 2006 (originally adapted from Doumenge and Mott, 1984).

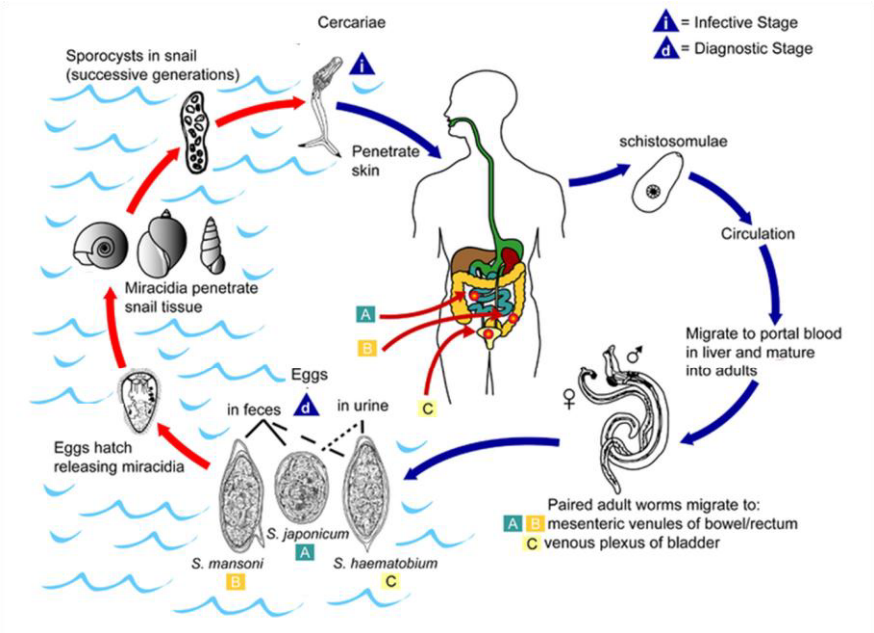

Figure 1.2. The life-cycle of *Schistosoma* spp. parasites. Adapted from online CDC resource (www.dpd.cdc.gov/dpdx/Schistosomiasis.htm). Blue arrows indicate stages to which the human host is exposed and red arrows indicate free-living aquatic stages and those affecting the intermediate snail host.

domestic 'water contact' activities including bathing, playing, water collection (Rudge *et al.* 2008) and occupational activities (e.g. fishing (Joseph *et al.* 2004a), car washing and sand harvesting (Black *et al.* 2009)). Cercariae invade percutaneously using proteolytic enzymes secreted from the acetabular glands to 'tunnel' through dermal barriers (Stirewalt and Kruidenier 1961). During invasion cercariae mature into schistosomula, which are exposed to a range of host tissues as they migrate from the skin via blood and lymphatic vessels to the lungs and are then transported in the blood to the urogenital (*S. haematobium*) or hepatic (*S. japonicum* and *S. mansoni*) veins. Adult schistosomes reside in these vessels and form long-term male-female pairs to produce eggs. Unlike the larval stages, which survive for hours (cercariae) or weeks (schistosomula) (Anderson and May 1992; Gryseels *et al.* 2006), adult worms have an average intra-host life-span of several years (the most recent estimate for *S. mansoni* is 3-10 years (Fulford *et al.* 1995) but estimates for *Schistosoma* spp. range from 6-11 years (Table 1.1)) allowing parasites to establish chronic infections.

Egg-stage schistosomes are either transmitted to the environment via passage into the urinary bladder (*S. haematobium*) or gastrointestinal (GI) tract (*S. mansoni* and *S. japonicum*). This is an immune-mediated process in murine infections (Doenhoff 1997; Fallon and Dunne 1999) and evidence that immunosuppressed individuals have impaired excretion of *S. mansoni* eggs suggests that this is also the case in human infections (Karanja *et al.* 1997). Schistosome eggs are also the predominant cause of host morbidity in chronic schistosomiasis since many become trapped in host tissues where they form fibrotic lesions called granulomas (Henri *et al.* 2002; Dessein *et al.* 2004; Coutinho *et al.* 2007). Accumulation of eggs occurs cumulatively and morbidity develops over many years (Dunne and Pearce 1999). Eggs that are successfully transmitted to the environment in urine (*S. haematobium*) or faeces (*S. mansoni* and *S. japonicum*) hatch in freshwater where they can infect intermediate host snails to maintain the life-cycle.

1.2.3 Distinctions between schistosome species

In this thesis (chapter 3-6) I have focused on *S. haematobium*, the most prevalent schistosome species in Africa (Chitsulo *et al.* 2000), but arguably the least studied. Unlike *S. mansoni* and *S. japonicum* for which genome sequences were recently published (Berriman *et al.* 2009; Zhou *et al.* 2009), the *S. haematobium* genome project is incomplete and its life cycle is also more difficult to maintain in laboratory animals than the other 2 species. The

mouse is a non-permissive host for *S. haematobium* (Moore and Meleney 1954; Cheever *et al.* 1983) and the experimental host repertoire is generally more restricted and patterns of infection more variable than those of experimental *S. mansoni* or *S. japonicum* (Meleney *et al.* 1953; Moore and Meleney 1954). Thus, much of what is known about schistosome immunobiology comes from *S. mansoni* (and to a lesser extent *S. japonicum*) infections in laboratory mice. However, distinctions in the *S. haematobium* life-cycle mean that urogenital schistosomiasis fundamentally differs from the intestinal forms of disease caused by *S. mansoni* and *S. japonicum*. Given that *S. haematobium* adult worms reside in the veins of the urogenital tract, deposition of *S. haematobium* eggs is associated with damage to these tissues and haematuria is a common symptom regardless of age, sex, nutritional status and environmental factors (Koukounari *et al.* 2007). If left un-treated *S. haematobium* infection can lead to hydroureter, hydronephrosis and bladder fibrosis (Gryseels *et al.* 2006) and is unique among the schistosome species in its link to the development of bladder carcinoma (reviewed by (Mostafa *et al.* 1999)) and female infertility (Kjetland *et al.* 2010). Large-scale human autopsy studies have also shown that *S. haematobium* eggs more often accumulate in host tissues than in their excreta relative to *S. mansoni* infections (Cheever *et al.* 1977), although the immunological relevance of these differences has not been investigated.

Murine infections also suggest that *S. haematobium* is more antigenically similar to the bovine parasite *S. bovis* than to *S. mansoni* as the 2 species can elicit heterologous immune responses to each other, but not to *S. mansoni* (Agnew *et al.* 1989a; Agnew *et al.* 1989b) and *S. haematobium* 28kDa glutathionine-S-transferase (GST, an abundant schistosomal enzyme and vaccine candidate antigen) shares greater sequence homology with *S. bovis* GST than *S. mansoni* or *S. japonicum* GST (Trottein *et al.* 1992a). Thus, despite similarities in their life cycle, epidemiology (chapter 1.2.4) and gene expression, the immune mechanisms identified in experimental and human infections with *S. mansoni* and *S. japonicum* should be extrapolated to *S. haematobium* with caution. Throughout this thesis I have referred to these studies where relevant and in particular where there is no equivalent for *S. haematobium*.

1.2.4 Epidemiology

Within endemically-exposed populations, the highest schistosome burdens are aggregated in a minority of individuals (Woolhouse 1994), an example of which is shown in Figure 1.3A. Furthermore, individuals with the highest parasite numbers before anti-helminthic treatment also

tend to accumulate high worm burdens after treatment (Bensted-Smith *et al.* 1987; Tingley *et al.* 1988; Etard *et al.* 1995), suggesting that certain individuals may: a) be genetically pre-disposed to infection (reviewed by (Quinnell 2003)), although evidence from field studies of schistosomiasis are mixed (Bethony *et al.* 2002; King *et al.* 2004), b) have greater exposure to infection due to their water contact behaviour (Chandiwana and Woolhouse 1991) and/or c) may not have developed protective immunological memory responses to schistosome antigens (Woolhouse and Hagan 1999). It has been known since the 1930s that schistosome worm burdens vary with age in endemic populations (Fisher 1934), suggesting that age-related changes also influence the risk of infection.

Cross-sectional population surveys suggest that worm burdens increase incrementally from birth with repeated exposure to infection, reach a 'peak' during late childhood/adolescence and are considerably lower (or absent) in adulthood (Fisher 1934; Anderson and May 1992; Agnew *et al.* 1996; Woolhouse 1998). Immune-mediated morbidities also tend to be aggregated in chronically infected school-age children who have high intensity infections, but a shorter history of exposure than adults (van der Werf *et al.* 2003; Garba *et al.* 2010). Thus, children tend to be considered susceptible (both to infection and associated morbidity), whilst adults appear more resistant to schistosomiasis. The basis of this age-infection pattern remains controversial since if exposure to infection occurs throughout life the relative importance of physiological changes with age and cumulative exposure to infection are difficult to extricate. Thus, on the one hand the cross-sectional decline in infection intensity in older children and adolescents tends to coincide with physiological, hormonal and behavioural changes at puberty that may alter susceptibility to infection (Fulford *et al.* 1998). However, comparisons between populations have shown that the age at which infection intensity begins to decline does not always occur at puberty and varies according to parasite transmission intensity (reviewed by (Woolhouse 1998)). Importantly, the age at which peak schistosome infection intensity occurs is at a younger age in populations with high transmission intensity than in those with low infection intensity (Mutapi *et al.* 1997; Woolhouse 1998).This pattern, known as the 'peak shift' (Figure 1.3B), indicates that exposure to a higher 'dose' of parasites from a young age, as would be the case in areas of high transmission, accelerates the development of resistance to infection/re-infection and thus worm burdens decline at an earlier age than in populations with limited exposure to infection (reviewed by Woolhouse in 1998). Variation in susceptibility/resistance between adults according to their history of exposure to infection also contradicts the view that the distribution of infection is intrinsically linked to age.

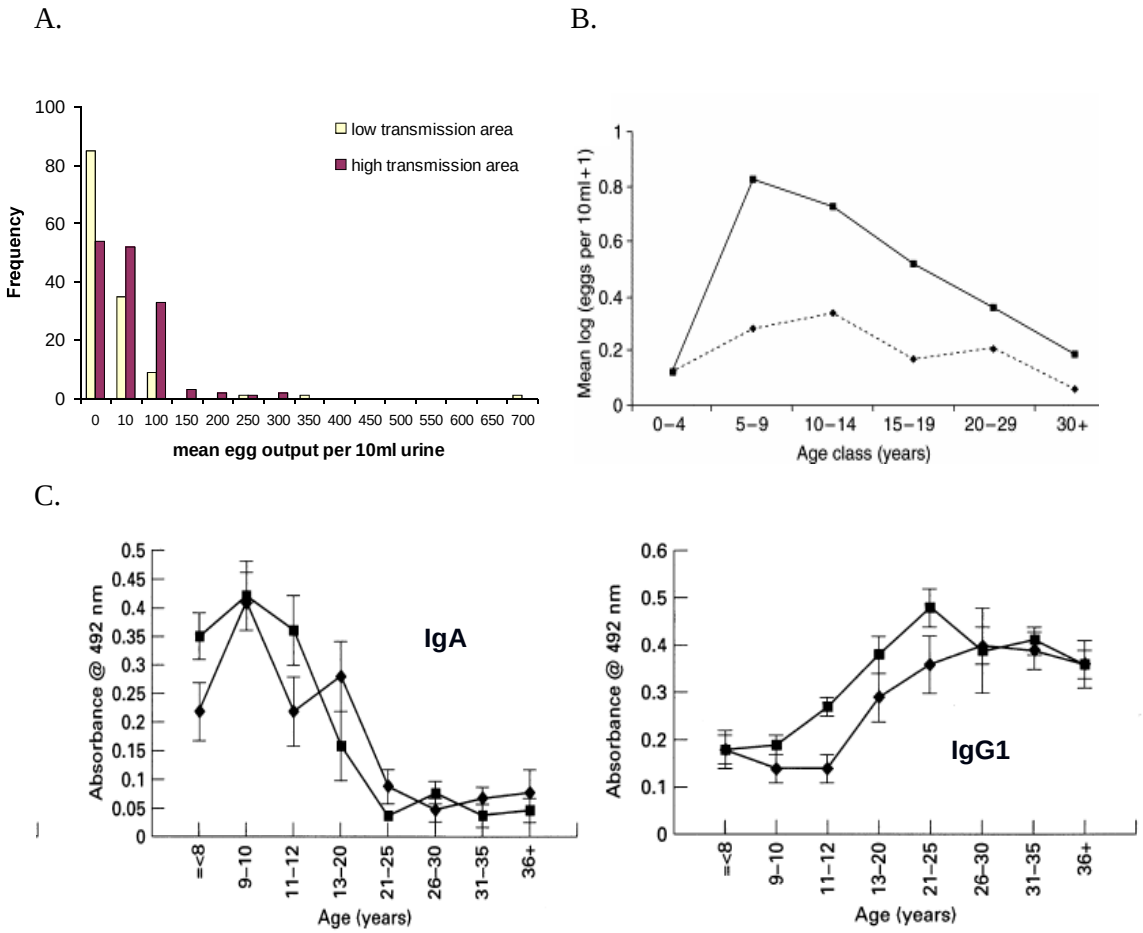

Figure 1.3. Epidemiological patterns in the distribution of human *S. haematobium* infections and parasite-specific immune responses. A) Frequency distribution of egg counts per 10ml urine, B) distribution of infection intensity by age and C) age-related changes in adult worm-specific serum IgA and IgG1 (quantified by ELISA) in 2 Zimbabwean villages with distinct transmission intensities. Yellow bars (A), dashed line (B) and diamonds (C) represent mean values of data collected from 133 participants from an area of low infection prevalence (33.8%). Red bars (A), solid line (B) and squares (C) represent mean values of data collected from 147 participants from an area of high infection prevalence (62.7%). Figures are reproduced from articles by Mutapi *et al*, 1997 (A and C) and Woolhouse, 1998 (B).

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This was first demonstrated in a study of Sudanese canal workers hyper-exposed to *S. mansoni* infection, which showed that those who had been occupationally exposed for over 10 years were more resistant to infection than newly recruited workers (Satti *et al.* 1996). More recently a direct comparison between two male cohorts occupationally exposed to *S. mansoni*, found that failure to cure infection following anti-helminthic treatment was less common in men with a long history of previous exposure to infection than in men who had been exposed more recently (Black *et al.* 2010).

1.3 Studying schistosome immunoepidemiology

Immunoepidemiology is the study of the patterns and relationships between infection and markers of the immune response at a population level and provides an important means of identifying correlates of resistance and susceptibility to disease (Hellriegel 2001). Identification of measurable changes in immune biomarkers (e.g. switching from IgG4 to IgE, IgA to IgG1 (Figure 1.3C) and IL-10 to IL-5 (Hagan *et al.* 1991; Mutapi *et al.* 1997; Mutapi *et al.* 2007b)) at the age of peak infection intensity provide the best evidence that the host immune response may drive the decline in worm burdens post-peak. In addition to age-related immunological changes within populations, comparison between populations reveals that isotype-specific antibody responses also follow 'peak shift' patterns (Figure 1.3C) (Mutapi *et al.* 1997). However, what is notably lacking from existing studies is a link between immunoepidemiological patterns of individual biomarkers (e.g. antibody and cytokine responses) and the more global changes in the host's immune response that they reflect. The latter may provide a better indicator of how protective immunity develops and which antigens are driving it in the context of considerable host heterogeneity and I have investigated this possibility in the immunoepidemiological study of *S. haematobium* described in chapters 3-6. Immunoepidemiological studies rely on collection of peripheral samples (e.g. venous blood, stool, urine) and thus the estimates of infection and immune responses come from indirect quantification. The disadvantage of using peripheral samples to assess immune responses is that they may not reflect tissue-specific levels of immunological analytes (Remick *et al.* 2000), however since adult schistosomes reside in the vasculature and chronic infection leads to systemic antigen exposure (Polman *et al.* 1998; van Lieshout *et al.* 1998) peripheral samples remain an effective indicator of immune variations. Furthermore, the major advantage of an immunoepidemiological approach over animal models of infection is that it allows immune responses to be characterised in their natural contexts,

incorporating host and parasite heterogeneity that are necessarily absent in the laboratory (Druilhe *et al.* 2002; Bourke *et al.* 2011).

1.3.1 Quantifying infection

Schistosomiasis is diagnosed and host infection intensity can be quantified by counting eggs present in the excreta. Microscopic examination of eggs in stool (*S. mansoni*, *S. japonicum* and STH) (Katz *et al.* 1972) or in filtered urine (*S. haematobium*)(Mott 1983) are the recommended methods of quantifying helminth infection intensity during human field studies since they are suitable for expedient processing of large numbers of samples in sites where specialist equipment and reliable electricity supplies are un-available (Montresor *et al.* 1998). In general egg counts are considered an indicator of adult worm burden, however accumulation of ectopic eggs (Cheever *et al.* 1977), temporal variations in egg production rates (van Etten *et al.* 1997; Hotez *et al.* 2008), reduced egg production per worm in high intensity infections (Cheever *et al.* 1977) and reduction in adult worm fecundity over time (Agnew *et al.* 1996) may all affect the correlation between egg output and adult worm numbers. Concerns have been raised that microscopy-based parasitological methods may lack the sensitivity to detect low intensity helminth infections (Kongs *et al.* 2001; Tarafder *et al.* 2010), however, analysis of repeat samples and repeat smears from each stool sample has been shown to significantly increase the sensitivity of detection via microscopy (Ebrahim *et al.* 1997).

Schistosome antigens can also be quantified in blood or urine via enzyme-linked immunosorbent assay (ELISA) or urine dipsticks (Nibbeling *et al.* 1997; Hassan *et al.* 1998; Kahama *et al.* 1998; Polman *et al.* 2000; Ugbomoiko *et al.* 2009; Standley *et al.* 2010; Stothard *et al.* 2011). Circulating antigens tend to reflect results obtained via microscopic egg counts (Polman *et al.* 2001; Hotez *et al.* 2008) although the strength of correlation between the 2 measures vary between studies (Agnew *et al.* 1996; Kahama *et al.* 1998; Hotez *et al.* 2008; Standley *et al.* 2010) and the efficacy of the antigen detection method is dependent on the choice of antigen (Kahama *et al.* 1998) and whether antigens are assessed in urine or serum samples (Hotez *et al.* 2008).

Another method is to use immunological indicators of parasite exposure (e.g. parasite-specific antibodies), which has proven particularly effective for identifying early exposure in

young children (Mutapi *et al.* 2011c; Stothard *et al.* 2011). However, in older people who have been exposed to but subsequently cleared schistosome infection these methods may be a less sensitive indicator of patent infections.

1.3.2 Quantifying immune responses

ELISA is a widely used means of measuring analytes in biological samples and has also been used extensively in chapters 3-6 of this thesis. Nearly all immunoepidemiological field studies conducted to date have used ELISA to quantify a range of immune biomarkers including cytokines, antibodies and cellular proteins. Total and antigen-specific antibody titres and non-specific circulating cytokine responses can be directly quantified in serum or plasma (Hagan *et al.* 1991; Fitzsimmons *et al.* 2004; Milner *et al.* 2010). However, since cytokines can be secreted in response to a range of stimuli and often act locally, systemic cytokines cannot be directly related to parasite-specific immune responses and may be present at very low or un-detectable levels.

Re-stimulation of whole blood (Scott *et al.* 2000; Scott *et al.* 2001; Joseph *et al.* 2004a; Mutapi *et al.* 2007b) or purified cell populations (Grogan *et al.* 1996a; van den Biggelaar *et al.* 2002; Silveira *et al.* 2004) prior to quantification is a useful means of assaying cytokine responses directly elicited by antigens of interest and also leads to much higher cytokine concentrations than in serum or plasma, allowing them to be more readily detected (Remick *et al.* 2000). Purification of cell populations from whole blood samples has the advantage of allowing the cellular source of assayed cytokines to be identified; however an important caveat is that these cells are not activated in isolation from other cell types *in vivo*. Assaying antigen-specific whole blood cytokine responses provides an effective alternative/complement to these studies since the physiological composition of cell types present in the vasculature is preserved and less sample processing is required, reducing the risk of artificially activating cells (Remick *et al.* 2000). Whole blood culture is also better suited to processing large numbers of samples during field studies since lower sample volumes are required than for cell purification and access to the equipment and reagents required for cell purification may be limited (Remick *et al.* 2000).

Cryopreserved cells can be further purified after collection and their phenotypes defined according to surface marker and intracellular cytokine expression assessed using flow

cytometry (Watanabe *et al.* 2007; Silveira-Lemos *et al.* 2008; Nausch *et al.* 2011). Alternatively purified cells can be used for functional assays *in vitro*, including assessment of proliferative responses to antigen stimulation (Grogan *et al.* 1996a; Medhat *et al.* 1998) and *in vitro* granuloma formation (IVGF) (El Ridi *et al.* 1997).

1.4 Schistosome antigens

Immunological changes during infection are closely linked to progression of the parasites through their life-cycle (Pearce and MacDonald 2002). This is clearly traceable in murine models where infected animals are exposed to each life-cycle stage sequentially. In schistosome endemic human populations however simultaneous exposure to adult worms and eggs during infection occurs in the context of repeated challenge with cercariae, which can make the association between parasite life-cycle stages and the host immune response more difficult to discern. In general human field studies have assessed immune responses to life cycle stage-specific crude homogenate preparations; cercarial antigen preparation (CAP), schistosomula antigen preparation (SAP), whole (adult) worm homogenate (WWH) and schistosome egg antigen (SEA). These preparations comprise stage-specific cocktails of parasite molecules including surface-exposed antigens, secreted molecules and those that are not exposed to the immune system by live parasites (e.g. cytosolic antigens) (Curwen *et al.* 2004). The advantage of using parasite antigens in their native (crude) form rather than recombinant antigens is that these preparations reflect the diverse range of parasite molecules present during infection, where parasite metabolism, death and the presence of cross-reactive antigens can influence the immune responses directed against each life-cycle stage. These preparations also incorporate post-translational modifications that may influence immune responses but are absent from recombinant antigens. Surprisingly, despite their wide use in immunoepidemiological studies, the immune responses to the different life-cycle stage-specific preparations has been compared in relatively few studies and early comparisons have never been up-dated to include markers associated with recently discovered cell types (chapter 1.5).

1.4.1 Cercariae

Cercarial antigens comprise a range of unique glucose and fucose carbohydrates found in the cercarial coating (glycocalyx) and tail (Nanduri *et al.* 1991; Xu *et al.* 1994) and the contents of the acetabular glands, which are actively secreted during invasion and enriched with glycans (Jang-Lee *et al.* 2007) proteases (e.g. cercarial elastases (Salter *et al.* 2002; Curwen *et al.* 2006)) and toxin-like molecules (Curwen *et al.* 2006). Notably the glycocalyx, tail and acetabular gland contents are lost during penetration and absent from later stages of the parasite (Samuelson and Caulfield 1985). Carbohydrate antigens derived from these structures are highly immunogenic in mice (Samuelson and Caulfield 1985; Hussein *et al.* 1997) and i*n vitro* stimulation with cercarial secretions have also been found to elicit a pro-inflammatory cytokine response in whole blood and peripheral blood mononuclear cells (PBMC) collected from an *S. mansoni* and *S. haematobium* co-endemic human population in Senegal (Dr. Joseph Turner, Liverpool School of Tropical Medicine, U.K., personal communication).

Pro-inflammatory responses of innate effector cells to cercarial antigens in the skin, which are well documented in murine schistosomiasis (Jenkins *et al.* 2005a; Paveley *et al.* 2009), may mediate the acute urticarial condition ('swimmer's itch') that is often noted in individuals who make only seasonal or one-off contacts with schistosome-infested water (Colebunders *et al.* 1995; Moore and Doherty 2005; Lambertucci 2010) . Swimmer's itch is rarely reported in endemic communities, which may reflect under-reporting (Appleton 1984), the relatively low severity of the condition (Lambertucci 2010) or development of tolerance to cercariae due to repeated exposure, as has been reported in repeatedly-infected mice (Cook *et al.* 2011). In the absence of repeat exposure, for example in tourists visiting endemic regions, elevated proliferative and cytokine responses to schistosome antigens can persist for years after initial exposure (Soonawala *et al.* 2011).

1.4.2 Schistosomula

Cercariae become classified as schistosomula after they enter the vasculature and thus this stage is associated with both migration and maturation of the parasite. Schistosomula tegument antigens are particularly immunogenic (more so than whole schistosomula antigen preparations) when cultured with PBMCs isolated from humans endemically-exposed to *S. mansoni* (Gazzinelli *et al.* 1983). However, whole schistosomula preparations and secreted vesicles have also been shown to elicit PBMC proliferation *in vitro* (Vieira *et al.* 1987).

Although schistosomula are of interest as an intermediate stage, they have not been investigated in this thesis since studies in *S. mansoni* suggest that over 80% of SAP constituents overlaps with WWH, whilst CAP and SEA express a more distinct protein repertoire (Curwen *et al.* 2004).

1.4.3 Adult worms

Despite their large size and prolonged exposure to circulating immune cells, there is little evidence for immune-mediated attrition of adult parasites during experimental infections (Meleney *et al.* 1953; Agnew *et al.* 1993). This may be due to direct modulation of systemic immune responses by adult parasites, which has been shown to occur in the absence of eggs in laboratory animals (Mangan *et al.* 2004; Smith *et al.* 2004; Mangan *et al.* 2006), or via sequestration of immunogenic antigens beneath their thick surface tegument (van Hellemond *et al.* 2006).

Separation of adult worm homogenates by 2-dimensional electrophoresis and proteomic analysis of their constituents has shown that the most abundant soluble proteins are cytosolic and therefore may not be exposed to immune recognition in live adult worms (Curwen *et al.* 2004). Consistent with these observations, Western blot screening of adult worm antigens with serum from humans endemically-exposed to *S. haematobium* infection suggest that not all adult worm antigens are constitutively exposed to the immune system during infection since the range and intensity of antigens recognised increases after treatment-induced killing of adult worms (Mutapi *et al.* 2005). The antigens that are exposed to immune-recognition prior to adult worm death are likely to be those present on the tegument surface or excreted/secreted, which is a restricted repertoire relative to the adult worm proteome as a whole (Curwen *et al.* 2004; van Hellemond *et al.* 2006). Adult worm tegumental extracts induce relatively poor *in vitro* proliferative responses when cultured with PBMC from endemically-exposed humans (Gazzinelli *et al.* 1983) and are less reactive with anti-sera from infected animals (Beisler *et al.* 1984) than whole worm antigen preparations. Together these observations suggest that tegumental antigens are not highly immunogenic *per se*, but immune responses to adult worms may be boosted by exposure to previously 'un-seen' adult worm constituents. This would be consistent with the hypothesis that release of antigens from dead or dying adult worms provides an important immune stimulus for protective immunity (Woolhouse and Hagan 1999) as has been shown in early animal models where

live adult worms do not activate the surrounding host tissues, but dead worms are rapidly cleared by infiltrating immune cells (Meleney *et al.* 1953).

Interestingly both the range and intensity of adult *S. haematobium* antigens recognised by human serum antibodies increases with age and infection intensity (Mutapi *et al.* 2008) and this is also the case for post-treatment antibody responses to *S. mansoni* antigens isolated from the adult worm tegument (Roberts *et al.* 1987). Thus, the duration and intensity of exposure to infection, rather than infection status alone appear to be key determinants of responsiveness to both tegumental and cytosolic adult worm antigens.

1.4.4 Eggs

Transition from the acute to chronic stage of schistosome infection can be clearly defined in murine models as the onset of oviposition (5-6 weeks post-infection) (Pearce and MacDonald 2002), which coincides with a large increase in circulating parasite antigens relative to the pre-patent stage of infection (Lei *et al.* 2009). Egg antigens are also clearly detectable in the urine (Nibbeling *et al.* 1998; Polman *et al.* 2000) and sera (Hassan *et al.* 1998; Polman *et al.* 2000) of infected humans. Thus, schistosome eggs are an abundant source of systemic antigen during infection. Furthermore, whilst ectopic eggs trapped in host tissues are essentially a 'dead end' in the parasite life cycle, detectable levels of egg antigens in urine after clearance of infection (Nibbeling *et al.* 1998) suggests that they continue to be a source of antigens for host immune cells.

Egg antigens are potent inducers of CD4+ T helper (Th) 2-type responses *in vivo* (described in chapter 1.5.3) and instigate a shift away from the pro-inflammatory responses that predominate during acute murine infections (Grzych *et al.* 1991; Pearce *et al.* 1991). Specific constituent antigens of homogenised eggs can also elicit Th2-type responses in human basophils, dendritic cells (DCs) and T cell cultures (Schramm *et al.* 2003; Everts *et al.* 2009). Egg glycans such as Lacto-N-fucopentanose III (LNFPIII) (Okano *et al.* 2001; Thomas *et al.* 2003) and Omega 1 (Schramm *et al.* 2003; Everts *et al.* 2009; Steinfelder *et al.* 2009) appear to be particularly important in this respect. Furthermore, crude egg but not adult worm antigens can directly induce IVGF by PBMCs collected from schistosome-exposed children (El Ridi *et al.* 1997), suggesting that the granulomatous response generated during infection is directly mediated by eggs.

1.4.5 Cross reactive antigens

In addition to the stage-specific expression of certain schistosome antigens cercariae, schistosomula, adult worm and egg stage parasites also share a range of antigens (Curwen *et al.* 2004; Jolly *et al.* 2007). Consistent with progression from one life-cycle stage to another, the soluble proteomes of adjacent stages are the most similar to each other (Curwen *et al.* 2004) and this may also be the case for the immune responses directed against them. The latter hypothesis is supported by a study which used rabbit anti-serum to show that antigens exposed on the adult worm tegument, but not in the gut, elicited antibodies that cross-react with egg antigens, but less so with cercarial antigens (Beisler *et al.* 1984).

Schistosome antigens also cross-react between species (Agnew *et al.* 1989a; Agnew *et al.* 1989b; Dean *et al.* 1996) and with antigens from other parasites, including *Plasmodium* spp. (Naus *et al.* 2003; Mutapi *et al.* 2007a) and STH (Correa-Oliveira *et al.* 1988; deNoya *et al.* 1996). Co-infection studies in humans suggest that schistosome immunopathogenesis may be influenced by the presence of other pathogens (Ganley-Leal *et al.* 2006; Geiger 2008; Wilson *et al.* 2008; Diallo *et al.* 2010), although whether these effects are due to antigen cross-reactivity is subject to debate (Reilly *et al.* 2008; Diallo *et al.* 2010).

1.5 Immune responses to schistosomes

Innate immune responses provide a first line of defence from pathogens. Cellular effectors of the innate immune system are non-clonal and interact with pathogens and their antigens directly via expression of pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs) (reviewed by Akira and colleagues (Akira *et al.* 2006)). Innate cells also process and present antigen to activate T cells, however, since helminths are too large to be internalised by phagocytic cells, presentation of their antigens is limited to their excreted/secreted molecules or those released when they die. Upon exposure to antigen CD4+ T cells are activated to selectively differentiate into alternative functional lineages: Th1, Th2, Th17 and T regulatory (Treg), as summarised in Figure 1.4. Chronic schistosomiasis was initially considered a ‘Th2’ disease, with the immune response characterised by Th2-associated effectors including production of

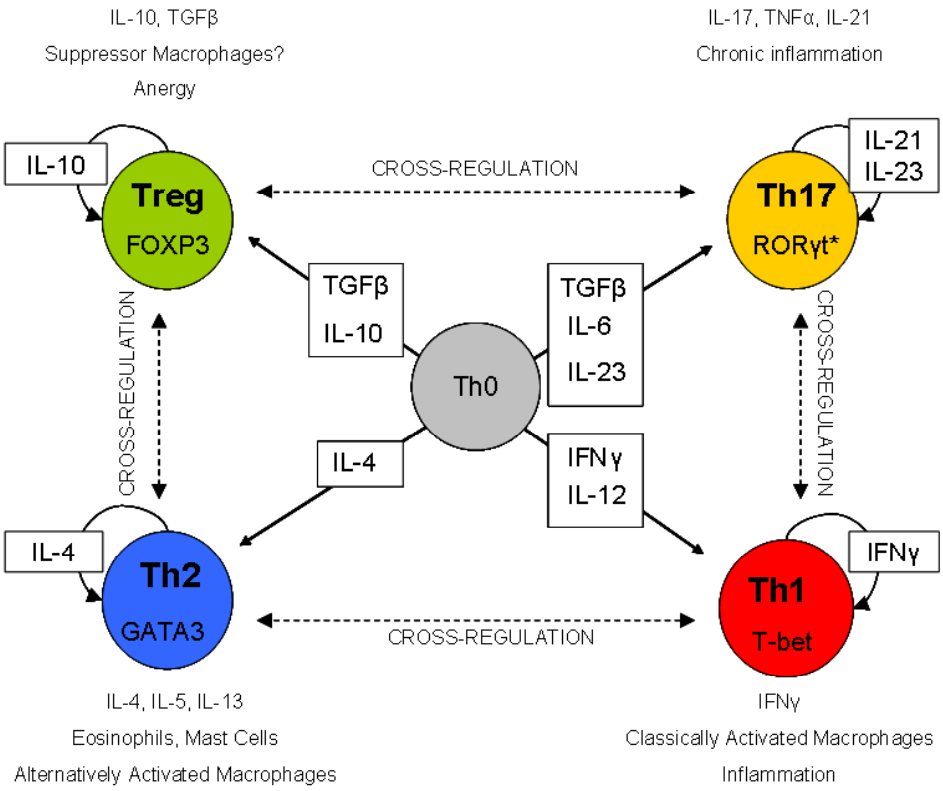


Figure 1.4. Summary of the major CD4+ T cell differentiation pathways following activation in the periphery. Cytokines and transcription factors involved in T cell polarisation are shown in boxes and within cells respectively. Effector cell types and cytokines associated with each CD4+ T cell phenotype are given adjacent to relevant cells. Th0 – naïve T cell, * RORγt – murine transcription factor, the human orthologue is RORC2. Figure reproduced from article by Bourke *et al*, 2011 (originally adapted from Deenick and Tangyne, 2007 and Diaz and Allen, 2007) with permission from the licensed content publisher.

interleukins (IL-)4, 5, 9, 10 and 13 (refer to reviews (Pearce and MacDonald 2002; Caldas *et al.* 2008)), secretion of Immunoglobulin (Ig)E and IgG4 isotypes by plasma cells (Hagan *et al.* 1991) and activation of downstream effector cells such as eosinophils (reviewed by (Klion and Nutman 2004)). These responses markedly differ from the Th1-type immune responses thought to typify infections with intracellular pathogens (the Th1-Th2 paradigm is reviewed and discussed elsewhere (Mosmann and Coffman 1989; Mosmann and Sad 1996; Allen and Maizels 1997)).

Whilst human field studies initially focused on the dichotomy between Th1 and Th2 cells and their associated effectors, cellular and cytokine responses rarely segregate according to these delineations (Contigli *et al.* 1999; Mduluza *et al.* 2001; Joseph *et al.* 2004a; Mutapi *et al.* 2007b; Milner *et al.* 2010). In particular, chronic schistosomiasis is characterised by immune hypo-responsiveness to parasite specific (Grogan *et al.* 1998b; Montenegro *et al.* 1999a; Silveira-Lemos *et al.* 2008) and non-parasite antigens (reviewed by Yazdanbakhsh *et al.* 2002), suggesting that both Th1 and Th2-type effector immune responses are modified by relentless exposure to parasite antigens, allowing infections to be tolerated for many years (Yazdanbakhsh and Sacks 2010). Regulatory mechanisms promoted during infection include secretion of suppressive cytokines, such as IL-10 and TGFβ, and expansion of regulatory cell populations, particularly Tregs (identified in human (Watanabe *et al.* 2007; Nausch *et al.* 2011) and murine (Stanworth and Smith 1973; Hesse *et al.* 2004; Wilson *et al.* 2005; Mo *et al.* 2007; Dittrich *et al.* 2008) schistosomiasis). Studies have also identified a range of surface-expressed and secreted molecules in a number of helminths species that can directly inhibit immune activation (reviewed extensively by Maizels and colleagues (Maizels *et al.* 1993; Maizels *et al.* 2001; Maizels and Yazdanbakhsh 2003; Maizels 2009; Maizels *et al.* 2009)), suggesting that parasites may actively perpetuate host hypo-responsiveness in the chronic stage of infection in order to facilitate intra-host survival.

The discovery of new cell populations including Treg and Th17 and the more recent description of IL-5 and IL-9 producing CD4+ T cells ('Th9'), which differentiate from Th2 cells in an IL-33 or TGFβ/IL-4 dependent manner respectively, has also prompted a re-evaluation of the classical Th1-Th2 paradigm (Dardalhon *et al.* 2008; Kurowska-Stolarska *et al.* 2008; Veldhoen *et al.* 2008). Furthermore, the CD4+ T cell axis can be regulated both by effector cytokines derived from CD4+ T cell populations themselves and innate and adaptive non-T cell populations (Hesse *et al.* 2004), including regulatory macrophages (Jenkins and

Allen 2010). Although the latter has been identified in murine schistosomiasis (Smith *et al.* 2004; Pesce *et al.* 2009), observations that human PBMC proliferation in response to schistosome antigens is enhanced by removing adherent/phagocytic cells (Todd, 1979), suggest that circulating non-lymphocytes may also play a regulatory role during human infection. Thus, immunoepidemiological studies that exclude non-T cells (e.g. via cell purification and cell-specific culture) during analysis may miss the important contribution of innate effector cells to anti-parasite responses.

1.5.1 Cytokine responses in helminth infection

Cytokines are soluble mediators of cellular immune responses and are secreted by all known cell types of the innate and acquired immune system. In most cases they act locally via autocrine or paracrine signalling however, where a cytokine producing cell is present in the circulation or a cytokine has a long half-life, these molecules can also have endocrine functions (Turnbull and Rivier 1999). Identification of cytokines has been instrumental in characterising cellular function and phenotype. This has been particularly effective for CD4+ T cell phenotypes (Mosmann *et al.* 1986; Cua *et al.* 2003; Veldhoen *et al.* 2008; Eyerich *et al.* 2009) as discussed above, however cytokine profiles have also been used to characterise subsets of other cell types, including macrophages (Pesce *et al.* 2009; Huang *et al.* 2010), DCs (Thomas *et al.* 2003) and B cells (Mangan *et al.* 2004). Extensive characterisation of different cytokines in humans and animal models has also made it possible to use cytokines as biomarkers of specific immune polarisations (Diaz and Allen 2007; Allen and Maizels 2011). Thus, although most immunoepidemiological field studies to-date have relied on analysis of individual cytokine responses, there has been a shift in murine studies towards characterising the type of immune responses elicited by helminth parasites rather than their individual constituents (Allen and Maizels 2011), which I hope to investigate further in this thesis.

1.5.1.1 Innate inflammatory cytokines

All known TLRs signal via MyD88, an intracellular signal transducer that activates secretion of systemic IL-6 by whole blood cells (Hayashi *et al.* 2001). In humans serum levels of IL-6 and TNFα are elevated during septic shock (Damas *et al.* 1992) and IL-6, IL-8 and TNFα are

secreted by PBMCs in response to viruses and their antigens (Schindler *et al.* 1990). Levels of these 3 pro- inflammatory cytokines are positively correlated when human PBMCs are cultured with agonistic antigens (e.g. endotoxin and phytohaemagluttanin (PHA)) (Schindler *et al.* 1990) .

TNFα was first discovered in mice in the 1970s as a cytotoxic factor predominantly produced by macrophages rather than lymphocytes (Carswell *et al.* 1975). In humans TNFα induces secretion of both IL-6 and IL-8 and these 3 cytokines are co-produced by activated human macrophages and DCs (Huang *et al.* 2010).

IL-6 functions range from a well characterised role in inducing the acute-phase protein response in the liver (Barton 1996) , activating human T cell proliferation (Tosato and Pike 1988) and enhancing antibody production by activated human B cells (Tosato *et al.* 1988). IL-6 is also essential for negative feedback regulation of TNFα secretion during endotoxin-induced inflammation (Schindler *et al.* 1990).

The principal role of IL-8 is as a chemoattractant of neutrophils, accumulation of which is a common feature of inflamed tissues (refer to reviews by Curfs and Oppenheim (Curfs *et al.* 1997; Oppenheim 2001)).

Despite the overwhelming focus on innate inflammatory cytokine responses in bacterial and viral infections, their role during human schistosomiasis is becoming increasingly apparent. Activation of innate inflammatory cytokines (and IL-10) by *S. haematobium* egg antigens appears to proceed via TLR2 ligation since blocking this receptor reduces PBMC TNFα, IL-6, IL-8 and IL-10 production *in vitro* independent of bacterial endotoxin-specific concentrations of these cytokines (van der Kleij *et al.* 2002a). However, PBMC TLR4-agonist and endotoxin-specific concentrations of TNFα, IL-6 and IL-8 are lower in *S. haematobium*-infected children than in un-infected children (van der Kleij *et al.* 2004), suggesting innate inflammatory cytokine responses to mitogenic stimuli are limited during chronic infection. Both TNFα and IL-6 are also readily produced by *in vitro* culture of human whole blood with S. *mansoni* SEA (and WWH to a lesser extent), however low parasite-specific IL-6 and TGFβ concentrations are associated with development of severe liver pathology (Wilson *et al.* 2008), suggesting that IL-6 feedback regulation and synergy with anti-inflammatory responses may be required to limit immunopathology. Innate

inflammatory cytokine responses to cercariae are yet to be characterised in human schistosomiasis, but are the focus of on-going studies (Dr. Joseph Turner, Liverpool School of Tropical Medicine, U.K., personal communication).

1.5.1.2 Th1-type cytokines

IFNγ production is one of the main phenotypic features of human and murine Th1 cells (Mosmann *et al.* 1986; Del Prete *et al.* 1991). IFNγ is mainly produced by T cells (CD4+, CD8+ and γδ) and natural killer (NK) cells in response to pro-inflammatory stimuli and is often up-regulated in the context of innate inflammatory cytokines (reviewed by Curfs *et al*, 1997). IFNγ ligation to its receptor on human mononuclear phagocytes promotes their activation via up-regulating surface receptors, anti-pathogen functions and cellular proliferation (Nathan *et al.* 1984; Celada and Schreiber 1985) and is a more effective activator of human macrophages than other pro-inflammatory cytokines (Nathan *et al.* 1984).

Despite being predominantly secreted by macrophages and B cells, IL-12 is also considered a Th1-type cytokine since it directly induces T cells to secrete IFNγ (Trinchieri 2003). The cross-talk between STAT4/T bet (intracellular markers of the Th1 phenotype), which are up-regulated by IL-12 and IFNγ signalling, and STAT6/GATA3 (intracellular markers of the Th2 phenotype) , which are up-regulated by IL-4 signalling, appears to be instrumental in determining CD4+ T cell polarisation (Szabo *et al.* 2000; Fields *et al.* 2002; Usui *et al.* 2003). However, whilst the broad association of IL-12 with 'Th1-type' immune responses holds true, the mechanisms of Th1 differentiation in human CD4+ T cells is less well defined than in the murine system (Rao and Avni 2000; Moingeon 2002). It has been shown that, of the 2 sub-units that make up IL-12 (p40 and p70), only p70 is specific for IL-12 since p40 is shared with IL-23 (p19 and p40) (see below) (Wilson and Maizels 2004), although not all studies make this distinction in their assays.

IL-2 is secreted by activated T cells and promotes cell growth and proliferation of T cells, B cells and NK cells (Curfs *et al.* 1997; Oppenheim 2001). Although IL-2 alone can promote Th2 proliferation, it up-regulates secretion of pro-inflammatory cytokines and IFNγ and in combination these cytokines promote a Th1-type environment (Mosmann and Coffman 1989; Oppenheim 2001). Initial characterisation of Th1 cells in humans confirmed that cells stably expressing IFNγ and IL-2 mRNA lacked expression of IL-4 and IL-5 and vice versa

(Del Prete *et al.* 1991) and IFNγ and IL-2 proteins are also co-expressed by human Th1 (Cousins *et al.* 2002).

In murine schistosomiasis mansoni IFNγ, IL-2 and IL-12, as well as IL-10, limit egg-induced granuloma formation (Wynn *et al.* 1994; Wynn and Cheever 1995; Wynn *et al.* 1995a; Wynn *et al.* 1995b), although IL-12 has also been shown to exacerbate granulomatous pathology in the absence of IFNγ (Wynn *et al.* 1995b). In human *S. haematobium* and *S. mansoni* infections, parasite-specific IFNγ is consistently higher in un-infected than in infected individuals prior to treatment (Mduluza *et al.* 2003; Silveira *et al.* 2004; Wilson *et al.* 2008) and appear to be actively down-regulated during infection (Grogan *et al.* 1998a; Montenegro *et al.* 1999a).

1.5.1.3 Th2-type cytokines

Polarisation of CD4+ T cells towards a Th2 phenotype was initially characterised in humans by the production of IL-4 and IL-5 (Del Prete *et al.* 1991), and IL-13 and IL-10 are also associated with Th2 responses in mice and humans (reviewed by Mosmann and Sad (Mosmann and Sad 1996)). IL-4 in particular appears to be a Th2-specific growth factor and an IL-2 alternative for the Th2 lineage in mice (Howard *et al.* 1982). IL-4 is produced by activated T cells, DCs, mast cells and basophils and can also activate these cells via the IL-4 receptor. Multiple functions have been attributed to IL-4 including induction of B cell proliferation, inhibition of Th1 polarisation and macrophage activation (Mosmann and Sad 1996; Curfs *et al.* 1997; Rao and Avni 2000) and regulation of pro-inflammatory cytokine production by circulating monocytes (Cluitmans *et al.* 1994; Chen and Manning 1996).

The predominant function of human IL-5 is to activate and attract eosinophils and basophils and this cytokine does not appear to affect B cell or T cell function directly (Sanderson 1992).

The functions of IL-4 and IL-13 show considerable overlap since they share a common receptor sub-unit (IL-4Rα), however IL-13 lacks the ability to directly induce Th2 polarisation since the full IL-13 receptor is not expressed by T cells (de Waal Malefyt *et al.* 1993). Both IL-4 and IL-13 inhibit pro-inflammatory cytokine secretion by human monocytes (de Waal Malefyt *et al.* 1993; Minty *et al.* 1993) and promote ‘alternative

activation' of murine macrophages involved in wound healing responses (reviewed by Jenkins and Allen (Jenkins and Allen 2010)). IL-4 and IL-13 are also elevated during local allergic responses in human mucosal tissues (Ghaffar *et al.* 1997), consistent with their role in activating goblet cell hyperplasia and mucus secretion, which are known to facilitate clearance of murine GI nematode infections (Finkelman *et al.* 2004).

IL-10 is a well characterised immunosuppressive cytokine and its direct inhibition of both Th1 and Th2 effector cytokine expression during schistosome infections has been demonstrated using IL-10 blocking antibodies in PBMC and human whole blood cultures stimulated with *S. mansoni* and *S. haematobium* antigens (Corrêa-Oliveira *et al.* 1998; Grogan *et al.* 1998a; Montenegro *et al.* 1999a; Mutapi *et al.* 2007b). In infected individuals of all ages parasite-specific IL-10 concentrations are higher than in their un-infected counterparts (van den Biggelaar *et al.* 2000; Silveira *et al.* 2004; Mutapi *et al.* 2007b; Caldas *et al.* 2008) or individuals who have not been exposed to infection (McManus *et al.* 1999), suggesting that IL-10 is a marker of infection and key regulator of immunopathogenesis during chronic infection. The negative association of IL-10 with exposure to *S. haematobium* and *S. japonicum* (McManus *et al.* 1999; Scott *et al.* 2001) and lower *S. haematobium*-specific IL-10 relative to effector cytokine responses in older, parasite-resistant individuals in endemic populations (Mutapi *et al.* 2007b) suggest that IL-10 responses are also influenced by age and exposure history.

Interestingly, although IL-10 is considered to be a Th2 cytokine, expression appears to be much lower in human CD4+ T cells expressing Th2 effector cytokines (particularly IL-5) or IL-2 (Cousins *et al.* 2002). Conversely, in Th2-polarising culture conditions, IL-4, IL-5 and IL-13 tend to be co-expressed by human CD4+ T cells (Cousins *et al.* 2002), which may reflect their genetic co-localisation within a Th2 gene cluster on chromosome 5 (van Leeuwen *et al.* 1989; Dolganov *et al.* 1996). This locus has been implicated in genetic predisposition to re-infection post-treatment in a pedigree analysis of *S. mansoni*-exposed individuals (Marquet *et al.* 1996). However, studies have also shown that IL-4 and IL-5 responses can dissociate in human nematode (Sartono *et al.* 1997) and trematode (Grogan *et al.* 1996a; Scott *et al.* 2000) infections, demonstrating that Th2-associated cytokines are not always co-expressed.

Since its discovery in mice in 2005 (Schmitz *et al.* 2005), a range of studies have supported a role for IL-33 in human Th2-type responses both as a chemoattractant for Th2 cells (Mannino *et al.* 1998) and also as a mediator of eosinophil, basophil and mast cell function (Herrstrom *et al.* 1997; Pecaric-Petkovic *et al.* 2009). Along with other epithelial cell-derived cytokines, IL-33 has also been linked to immune responses during Th2-mediated allergy (reviewed by (Smits *et al.* 2010)). Murine infections with *Trichuris muris* are associated with an increased expression of IL-33 mRNA in the caecum (Humphreys *et al.* 2008) however (at the time of writing) no studies have investigated expression of this cytokine in any human helminthiases.

1.5.1.4 Th17-type cytokines

The identification of the Th17 lineage and its role in chronic inflammatory diseases has led to growing interest in the function of Th17 cells in other contexts. In murine and human T cells IL-17 production is a definitive marker of Th17 cells (Wilson *et al.* 2007; Volpe *et al.* 2008). IL-17 secreted by activated T cells is a potent chemoattractant for neutrophils and IL-17 has been shown to perpetuate tissue inflammation in mice (Liang *et al.* 2007; Hammerich *et al.* 2011). In addition IL-23 and IL-21 appear to perpetuate IL-17 secretion by CD4+ T cells (Hoeve *et al.* 2006) and, unlike murine Th17 differentiation which is driven by a combination of TGFβ and IL-6, IL-23 appears to be the principal cytokine promoting Th17 differentiation in humans (Hoeve *et al.* 2006; Wilson *et al.* 2007). Activated dendritic cells and macrophages are major sources of IL-23 (Oppmann *et al.* 2000; Verreck *et al.* 2004) and, in addition to promoting Th17 differentiation, IL-23 also up-regulates IFNγ expression by human T cells *in vitro* (Hoeve *et al.* 2006).

Despite promoting IL-17 secretion, the association of IL-21 with Th17-type responses is less clear-cut than for IL-23 and IL-17. In mice IL-21 is preferentially produced by Th2 relative to Th1 cells (Wurster *et al.* 2002). IL-21 and IFNγ also appear to regulate each other since IL-21 is transcriptionally repressed by T-bet, a Th1-associated transcription factor (Mehta *et al.* 2005). However, whilst IL-21 can inhibit IFNγ secretion at an early stage of T cell priming (Wurster *et al.* 2002), it can also up-regulate expression of Th1-associated markers on activated peripheral T cells (Strengell *et al.* 2002). Furthermore, expression of IL-21 and IL-21 receptor during both Th1 and Th2-polarised responses (Pesce *et al.* 2006) and the distinct genetic locus of IL-21 relative to Th2-type cytokines (Mehta *et al.* 2005) suggests

that it is not restricted to a single CD4+ T cell lineage, at least in mice. Clearly further investigation of the Th17 phenotype is required to test the relevance of these experimental observations in humans. In particular, it remains to be seen whether associations between IL-21 and Th17 responses in humans (but not in mice) reflect fundamental differences in human and murine immunology or simply a lack of study.

There is growing evidence that Th17 cells may contribute to pathological immune responses in murine schistosomiasis. Recent studies of murine *S. mansoni* infection suggest that IL-23p19 promotes hepatic pathology in response to injected SEA via up-regulating IL-17 expression in granulomas (Rutitzky *et al.* 2008). A recent pilot study in human filariasis also found that IL-17, IL-21 and IL-23 mRNA expression by PBMCs after stimulation with *Brugia malayi* antigens was elevated in individuals with lymphedema relative to their asymptomatic counterparts (Babu *et al.* 2009). Only one study has measured Th17-type cytokine responses in human schistosomiasis and showed that the prevalence of detectable parasite non-specific IL-17 and IL-23, but not IL-21 in plasma, was greater in *S. haematobium* egg-positive relative to egg-negative participants (Milner *et al.* 2010).

1.6 Protective immunity

Since schistosome infections affect multiple host tissues and their antigens circulate systemically, protective immunity must comprise both effector mechanisms to clear infection and regulatory mechanisms to limit immune-mediated pathologies (Allen and Maizels 2011). Thus protective immunity can be classified via several overlapping mechanisms ; a) complete elimination of parasites (sterile immunity), b) tolerance of patent infection but resistance to *de novo* infection (non-sterile immunity, also called 'concomitant immunity'), c) responses that reduce egg production or viability (anti-fecundity or anti-transmission immunity) and d) reduction of immunopathology (tolerance). I will refer to the former 3 processes collectively as 'anti-parasite immunity' and the latter as 'anti-pathology immunity'. The high prevalence of schistosome infections in endemic populations suggests that sterile immunity is rarely generated. However, epidemiological patterns of infection (see chapter 1.2.4) indicate that non-sterile immunity, though slow to develop, does occur with cumulative exposure (Woolhouse 1998; Yazdanbakhsh and Sacks 2010). Conversely, since the majority of schistosome infections do not result in mortality, it seems likely that hosts develop a degree of tolerance to low-level infections in order to limit tissue damage (Dessein

et al. 2004) (Yazdanbakhsh and Sacks 2010) and thus even infected individuals do not lack immune-mediated protection.

Immunological correlates of anti-parasite and anti-pathology immunity identified to-date are summarised below, however the molecular mediators of these processes for endemically-exposed hosts have not been conclusively defined. Furthermore, as highlighted in recent reviews (Diaz and Allen 2007; Jenkins and Allen 2010; Allen and Maizels 2011), the balance between pleiotropic and redundant cytokines and their associated cellular effectors is dynamic and a single molecule is unlikely to confer protective immunity in isolation or at all stages of a life-time spent exposed to parasite challenge.

1.6.1 Anti-parasite immunity

In humans, resistance to high intensity schistosome infections is typically attributed to elevated titres of parasite-specific IgE whilst IgG4 is considered a marker of infection and susceptibility both before and after treatment (Hagan *et al.* 1991; Rihet *et al.* 1991; Viana *et al.* 1995; Grogan *et al.* 1996b; Al-Sherbiny *et al.* 2003; Acosta *et al.* 2004; de Moira *et al.* 2010). For *S. haematobium,* circulating IgE responses can be induced by adult worm or egg antigens (Hagan *et al.* 1991) . The association between IgE and anti-parasite immunity may reflect the ability of IgE to bind to high affinity receptors on eosinophils facilitating antibody-dependent cell-mediated cytotoxicity (ADCC) (David *et al.* 1980; Gounni *et al.* 1994; Ganley-Leal *et al.* 2006). Eosinophil expression of FcεR is elevated during infection and eosinophil counts are associated with resistance to re-infection with *S. mansoni* (Ganley-Leal *et al.* 2006). Incubation of human eosinophils with schistosomula leads to direct killing of larval parasites (Butterworth *et al.* 1974; Butterworth *et al.* 1975; Hagan *et al.* 1985) and eosinophil granules are toxic to schistosomula *in vitro* (David *et al.* 1980; Ackerman *et al.* 1985), although a variety of antibody isotypes may mediate this process (Khalife *et al.* 1989; Dunne *et al.* 1993). In addition to IgE a number of other antibody isotypes have been associated with resistance to schistosome infection (e.g. IgG1 (Mutapi *et al.* 1997) and IgA (Acosta *et al.* 2004)), the schistosome antigen-specificity of IgE from endemically-exposed individuals overlaps with that of IgA, IgG1 and IgG4 (Mutapi *et al.* 2011a)(and potentially others) and IgE responses often correlate with other antibody isotypes during infection (Viana *et al.* 1995), which may more accurately reflect variation in infection intensity, exposure and treatment history than IgE titres (Webster *et al.* 1997a). Importantly, as noted

for cytokine responses (Mutapi *et al.* 2007b), reciprocal relationships between different isotypes rather than levels of individual antibodies appear to be important determinants of resistance/susceptibility to infection (e.g. the ratio between IgE and IgG4 (Hagan *et al.* 1991; Demeure *et al.* 1993) and IgG1 and IgA (Mutapi *et al.* 1997)).

Effector immune responses elicited by adult parasites but targeting cercariae have been proposed as a mechanism for limiting accrual of parasites in the context of on-going infection (concomitant immunity). This was originally shown in experimental *S. mansoni* infections of rhesus monkeys, where implantation of adult parasites induced resistance to cercarial invasion but prior-exposure to irradiated cercariae, which do not reach maturity, did not (Smithers and Terry 1967). That these mechanisms do not target adult parasites may be due to the reduced susceptibility of parasites to ADCC-mediated killing as they mature, although this has only been shown in murine studies *in vitro* (Moser *et al.* 1980; Ahmed *et al.* 1997). Paradoxically anti-larval immunity may not be elicited by larval parasites due to short-term exposure of larval peptides during invasion (Fitzsimmons *et al.* 2007), relatively low expression of 'protective' antigens compared to adult parasites or because immune responses in the skin are biased towards immunogenic carbohydrate antigens that cannot elicit memory T cell responses (Woolhouse and Hagan 1999). In human helminth infections the stimulus and target of anti-parasite immunity are difficult to distinguish since resistance to high worm burdens can only be assessed via egg output and circulating antigen levels. However, development of concomitant immunity to infection is corroborated by the predictions of mathematical models of infection (Woolhouse 1994) and empirical evidence for the development of immune-mediated resistance to the larvae of tissue-dwelling nematodes in endemically-exposed populations (e.g. PBMC-mediated resistance to L3 invasion in *Onchocerca volvulus* infection in Cameroon (MacDonald *et al.* 2002) and peripheral antibody-dependent immunity to *W. bancrofti* larvae with age in Papua New Guinea (Day *et al.* 1991a; Day *et al.* 1991b)).

Both eosinophilia and IgE responses in human schistosomiasis are linked to Th2-type cytokine responses and particularly IL-5 (Dutra *et al.* 2002; Ganley-Leal *et al.* 2006; Walter *et al.* 2006; Silveira-Lemos *et al.* 2008). A variety of immunoepidemiological studies have implicated Th2-type cytokines in anti-*S. haematobium* immunity, including higher adult worm-specific PBMC IL-4 and IL-5 in individuals resistant to re-infection (Medhat *et al.* 1998) and increased IL-5 responses to WWH with age in un-treated populations (Mutapi *et*

al. 2007b). However, the latter study found that reciprocal changes in IL-10, levels of which peak in young children who are most susceptible to infection and can suppress adult worm-specific IL-5 responses *in vitro*, may be a more important determinant of resistance to *S. haematobium* infection than levels of individual effector cytokines (Mutapi *et al.* 2007b). The importance of effector: regulatory immune responses in the development of resistance to infection with age is supported by age-related changes in the relationship between circulating effector T cells (Teff):Treg ratios and infection intensity in *S. haematobium*-exposed cross-sectional populations (Nausch *et al.* 2011) and observations that high levels of IL-10 are associated with an increased the risk of re-infection after treatment of chronically-exposed children (van den Biggelaar *et al.* 2002). A number of studies have also suggested that *S. haematobium* and *S. mansoni*-specific Th1-type cytokines are associated with resistance to infection (Mduluza *et al.* 2003; Silveira *et al.* 2004; Wilson *et al.* 2008) and re-infection post-treatment (Grogan *et al.* 1998b; El Ridi *et al.* 2001), suggesting that protective immunity in humans may constitute a mixed rather than Th2-polarised effector phenotype.

Evidence for a reduction in egg counts in the absence of adult worm clearance during natural *S. haematobium* infections comes from observations that egg counts, but not circulating antigen levels, decline with age (Agnew *et al.* 1996). This effect was not observed in *S. mansoni*-infected populations (Agnew *et al.* 1996). However, since egg excretion can also be reduced by sequestration of eggs in tissues (Cheever *et al.* 1977) and immunocompromisation (Karanja *et al.* 1997) it is unclear whether these observations reflect a genuine reduction in fecundity or species-specific variations in egg transmission with time post-infection. Due to the association between parasite eggs and morbidity in chronic schistosomiasis, anti-pathology immunity may also be achieved by reducing adult worm fecundity.

1.6.2 Anti-pathology immunity

Schistosome morbidity is characterised by immune hyper-reactivity to parasite antigens, particularly those of eggs. For example in human *S. haematobium* infections both SEA-specific TNFα and the ratio of SEA-induced PBMC TNFα to IL-10 are higher in subjects with urinary tract morbidity than in those without morbidity (Wamachi *et al.* 2004). High levels of parasite-specific IL-5 after treatment are also a risk factor for subsequent development of microhaematuria (van den Biggelaar *et al.* 2002). Elevated eosinophil

activation markers and expression of SEA and WWH-specific TNFα, IL-4 and IL-5 are also associated with periportal fibrosis in chronic *S. mansoni* infections (Silveira-Lemos *et al.* 2008). These observations suggest that, despite their association with anti-parasite immunity, effector cytokine responses are suppressed as a counter-balance to acute reactivity to parasite antigens. This may be particularly the case in young children where regulatory responses, including Tregs (Nausch *et al.* 2011) and parasite-specific IL-10 (Silveira *et al.* 2004), are positively correlated with infection intensity.

In addition to Treg and IL-10-mediated immunoregulation, activation of multiple cell types in response to systemic antigen may induce cross-regulation between effector cells. For example in chronic *S. mansoni* infections, Th2-mediated damage may be limited by Th1-type (Henri *et al.* 2002; Dessein *et al.* 2004) and innate inflammatory cytokines (TNFα and IL-6 (Booth *et al.* 2004; Wilson *et al.* 2008). Conversely, Th2-type responses appear to mitigate pro-inflammatory pathologies since liver damage and mortality are associated with an absence of Th2-associated biomarkers (Dunne *et al.* 2006; Wilson *et al.* 2008). Most recently T bet and IFNγ -dependent processes have been shown to reduce egg-specific Th17-type cytokine responses and TNFα in murine infection models (Rutitzky *et al.* 2008; Rutitzky *et al.* 2009), although the relevance of this for human anti-pathology immunity is yet to be tested.

1.7 Control of schistosomiasis

The World Health Organisation (W.H.O.) currently advocates 3 main interventions to control parasitic helminthiases: 1) chemotherapy to reduce morbidity due to high worm burdens, particularly in children, 2) improved sanitation as a means to reduce parasite transmission and 3) health education to encourage populations to modify their water contact behaviours and thus reduce both transmission and the risk of re-infection post-treatment (Montresor *et al.* 2002). There is no effective vaccine for schistosomiasis, although this remains an attractive prospect for long-term control of infections, particularly where behavioural modifications are un-realistic (e.g. in very young children and communities without access to sanitation) and access to chemotherapy is sporadic (Chitsulo *et al.* 2000).

1.7.1 Mass drug administration

Mass anti-schistosome treatment of all community members regardless of age, sex or infection status is recommended where infection prevalence is 50% or over as assessed by parasitological analysis in sentinel primary schools (classified as 'high prevalence'), targeted treatment of school-age children is recommended if infection prevalence is between 20 and 50% (moderate prevalence) and treatment of infected individuals or those with visible haematuria is recommended for communities where prevalence is below 20% (Montresor *et al.* 1998; W.H.O. 2006). The recommended treatment for schistosome infections is a single 40mg/kg body weight dose of the anti-schistosomal drug praziquantel or 2 doses of 15-30mg/kg of oxamniquine, which is effective only against *S. mansoni* (Montresor *et al.* 1998; Montresor *et al.* 2002). This summary will focus on praziquantel, which remains the most effective and widely used anti-schistosomal drug (Danso-Appiah *et al.* 2008).

Mass treatment with praziquantel is highly effective at clearing infection with studies reporting cure rates (i.e. no detectable infection) of over 80% 6 weeks after treatment (Tchuem Tchuente *et al.* 2004; Midzi *et al.* 2008a). Much lower cure rates have been reported where post-treatment parasitological assessments were made within 6 weeks of treatment (Saathoff *et al.* 2004; Tchuem Tchuente *et al.* 2004; Sissoko *et al.* 2009) although this appears to be due to a lag in excretion of eggs from tissues rather than a delayed effect against adult worms (Tchuem Tchuente *et al.* 2004; Guidi *et al.* 2010). Delayed excretion of eggs relative to adult worms after treatment is also consistent with post-treatment studies of *S. mansoni* where levels of adult worm antigen in urine decrease rapidly (within 1 week), but egg antigens were still detectable in urine 6 weeks after successful clearance of infection (Nibbeling *et al.* 1998). Importantly, even in studies reporting low rates of complete cure, the overall reduction in *S. haematobium* egg counts within treated cohorts were found to be consistently high (over 80%) and treatment-related side-effects mild or absent (Saathoff *et al.* 2004; Tchuem Tchuente *et al.* 2004; Midzi *et al.* 2008a; Sissoko *et al.* 2009). Although concerns have been raised that mass anti-helminthic treatment programs may promote drug resistance (Redman *et al.* 1996), longitudinal treatment-re-infection studies provide no evidence for reduced praziquantel efficacy against schistosome infections after repeated doses (Gryseels *et al.* 2001; Black *et al.* 2009). Longitudinal follow-up studies in *S. mansoni* affected populations also suggest that the severity of liver fibrosis is reduced in the majority of treated individuals (Martins-Leite *et al.* 2008; Rahoud *et al.* 2010). Furthermore, although

children aged under 5 years are currently excluded from mass treatment programmes due to concerns about side-effects and the disputed assumption that this age group does not harbour sufficient levels of infection to cause significant morbidity, preliminary studies provide evidence of both exposure to infection and morbidity in this age group(Opara *et al.* 2007; Mutapi *et al.* 2011c; Stothard *et al.* 2011). A recent safety-efficacy trial conducted in parallel with the study described in chapters 2-6 has also proven that children aged under 5 are tolerant of praziquantel treatment (Mutapi *et al.* 2011c).

In areas where schistosomiasis and STH are co-endemic, praziquantel is often co-administered with albendazole. In contrast to praziquantel, albendazole has only limited solubility and its activity is restricted to parasites in the GI tract (Marriner *et al.* 1986; Urbani and Albonico 2003). No synergy between albendazole and praziquantel activity has been observed in the clearance of schistosome or STH infections (Sirivichayakul *et al.* 2001; Pengsaa *et al.* 2004) and no increase in adverse side-effects has been reported for combined treatment (Olds *et al.* 1999).

1.7.2 Praziquantel mode of action

Praziquantel appears to act via paralysing adult parasites and disrupting their tegument, which has been visualised *in vitro* via electron microscopy (Becker *et al.* 1980). However, only one study to-date has conclusively identified a molecular target of praziquantel, the *S. mansoni* myosin light chain, which is abundantly expressed by adult worms and schistosomules and to a lesser extent by cercariae (Gnanasekar *et al.* 2009). Murine studies suggest that the schistosomicidal activity of praziquantel relies on activation of the host immune response (Brindley and Sher 1987; Doenhoff *et al.* 1987), which may be promoted by treatment-induced exposure of antigens on the adult worm surface (Harnett and Kusel 1986; Redman *et al.* 1996), including GST (Dupre *et al.* 1999).

Although the principal *in vivo* target of praziquantel appears to be adult worms and it has been shown that immature *S. mansoni* parasites are less susceptible to praziquantel-mediated damage in murine infection models (Andrews 1985; Sabah *et al.* 1986; Shaw 1990), immature worms are still damaged even at sub-curative doses and even adult worms vary in their susceptibility (Shaw 1990). *In vitro* studies also show that praziquantel can reduce cercarial tail shedding, egg hatch rates and miracidial motility in laboratory *S. mansoni*

isolates (Liang *et al.* 2001). Furthermore, whilst researchers hypothesise that eggs and larval stages may be sequestered from circulating praziquantel relative to adult *S. mansoni* in the hepatic veins (Sabah *et al.* 1986; Picquet *et al.* 1998), intra-dermal and intra-muscular injection of the drug reduces migration of *S. mansoni* cercariae and schistosomula in mice (Flisser *et al.* 1989).

Since orally administered praziquantel is absorbed into the bloodstream within 2 hours and approximately 70% is excreted in urine 24 hours later (Njomo *et al.* 2010), it is unclear whether its active components can access parasites in human tissues. However, orally-administered doses cause a rapid release of *S. mansoni* egg enzymes (Xu *et al.* 1988) and a reduction of tissue egg viability in murine schistosome infections (Giboda and Smith 1994). Treatment has been also been shown to reduce the viability of *S. haematobium* eggs excreted up to 7 weeks (Guidi *et al.* 2010) and increase *S. haematobium* egg-specific serum antibody titres relative to pre-treatment levels up to 18 and 36 weeks (Mutapi *et al.* 1998b) after successful treatment in human populations.

1.7.3 Effects of praziquantel on the immune response

Praziquantel treatment is known to alter human immune responses (Barsoum *et al.* 1982; Butterworth *et al.* 1985; Colley *et al.* 1986; Roberts *et al.* 1987; Medhat *et al.* 1998; Mutapi *et al.* 1998b; Mutapi *et al.* 2002; van den Biggelaar *et al.* 2002; Fitzsimmons *et al.* 2004; Joseph *et al.* 2004b; Mutapi *et al.* 2005; Reimert *et al.* 2006) and these effects may be due to activation by the release/exposure of parasite antigens (discussed above) and/or a rebound in effector immune responses due to the removal of immunosuppression mediated by live parasites. The latter is supported by the observation that the proportion of Teff:Tregs increases 8-14 months after treatment of *S. mansoni* relative to pre-treatment levels (Watanabe *et al.* 2007) and increased antibody responses to host anti-nuclear antigen (a marker of immune reactivity) 6 months after clearance of *S. haematobium* infection (Mutapi *et al.* 2011b). A boost in immune responses following treatment has been demonstrated as early as 24 hours after treatment of *S. mansoni* in humans, where plasma levels of eotaxin, IL-5 and IL-10 are elevated leading to eosinophilia at 3 weeks post-treatment (Reimert *et al.* 2006). PBMC proliferative responses to schistosome antigens are also enhanced by treatment (Barsoum *et al.* 1982; Colley *et al.* 1986; Feldmeier *et al.* 1988) and serum titres of *S. mansoni* and *S. haematobium* adult worm-specific antibodies (van den Biggelaar *et al.* 2002;

Walter *et al.* 2006) and *S. haematobium* egg-specific antibodies (Mutapi *et al.* 1998b) are also boosted by clearance of infection.

Treatment also induces changes in cellular cytokine responses, although there is considerable variation between studies both in methodology and findings. In an *S. mansoni*-exposed population, Th2 cytokine responses in plasma were elevated within 21 days of treatment (Fitzsimmons *et al.* 2004) and adult worm, but not egg-specific, Th2-type whole blood cytokine levels were increased 7 weeks after treatment relative to pre-treatment levels (Joseph *et al.* 2004b). However, Th1 and Th2-type whole blood cytokines responses to both SEA and WWH were found to be elevated post-treatment in a separate *S. mansoni*-exposed cohort, although cultures were conducted for considerably longer (Tweyongyere *et al.* 2009). Studies investigating whole blood cytokine responses to *S. haematobium* antigens suggest that Th2-type responses are differentially affected by treatment (Scott *et al.* 2000) a phenomenon that has also been observed in CD4+ T cell cytokine responses to *S. haematobium* antigens (Grogan *et al.* 1996a). PBMC cytokine studies after *S. haematobium* treatment broadly support the findings of *S. mansoni* studies that levels of Th2-type cytokines increase after treatment (van den Biggelaar *et al.* 2002; Mduluza *et al.* 2009), although it is noteworthy that the former study compares responses between an un-treated and treated cohort rather than the responses of the same individuals before and after treatment and reports only cytokine responses elicited by adult worm antigens. Importantly, no studies have investigated whether other cellular phenotypes or the balance between them are influenced by praziquantel treatment.

The protective efficacy of treatment-induced changes to the host immune response remains a subject of debate, particularly since even repeatedly treated cohorts remain susceptible to re-infection (van den Biggelaar *et al.* 2002; Guidi *et al.* 2010) and there is a resurgence of schistosome-associated pathologies at a population level after treatment has stopped (Wagatsuma *et al.* 1999; van den Biggelaar *et al.* 2002). However, longitudinal treatment-re-infection surveys indicate that whilst some individuals are re-infected rapidly others remain free from infection for prolonged periods and a number of studies have identified an association between post-treatment immune responses, particularly parasite-specific antibody titres (Hagan *et al.* 1991; Mutapi *et al.* 1999; Caldas *et al.* 2000) and cellular proliferative responses (Colley *et al.* 1986), and a reduced risk of re-infection. To date the results of studies investigating the association between cytokine responses and the risk of re-

infection have been inconsistent. For example, a study of adolescent boys found an association between adult worm-specific PBMC IL-4 and IL-5 and resistance to *S. haematobium* re-infection in Egypt (Medhat *et al.* 1998), whilst in a cohort of 5-14 year olds in Gabon adult worm-specific PBMC IL-10 was associated with an increased risk of *S. haematobium* re-infection and IL-5 was not associated with resistance to re-infection (van den Biggelaar *et al.* 2002). Variation in host age (9-35 years) and exposure have also confounded identification of a significant association between *S. mansoni*-specific cytokine responses and subsequent re-infection (Roberts *et al.* 1993). These inconsistencies may reflect variation in age ranges, gender and water contact activities between the study cohorts, but may also be due to the limited range of cytokines (6 or less) investigated and analysis of these responses one at a time, rather than as constituent parts of an immune profile. Thus a more comprehensive analysis of post-treatment cytokine responses is required to assess their relationship with re-infection.

1.8 Vaccination

1.8.1 Vaccine development

Since studies in the late 1970s found that up to 70-80% resistance to cercarial challenge could be induced in mice by pre-exposure to a single dose of radiation-attenuated (RA) cercariae (Minard *et al.* 1978; Murrell *et al.* 1979), significant efforts have been made to identify immunogenic parasite antigens that can induce equivalent efficacy. These have included isolation of sero-reactive molecules from RA cercariae (Richter and Harn 1993), probing the schistosomulum surface with monoclonal antibodies (Pierce *et al.* 1987) and selecting molecules according to recognition by serum antibodies from children endemically exposed to schistosomes but resistant to re-infection after treatment (Dessein *et al.* 1988). In 1998 a meeting in Egypt led to the identification of the major vaccine candidate antigens capable of inducing protective immunity in animal models of *S. mansoni*, which were glutathionine-S-transferase 28kDa (GST), Paramyosin (Sm97), Irv5, Triose-phosphate isomerase (TPI), Sm23 and Sm14 ((Bergquist and Colley 1998). Independent laboratory trials of these vaccine candidate antigens in animal models were conducted, but the results have never been published (Wilson and Coulson 2006). Subsequent correlate studies of the immune responses elicited by the same antigens (and others) in endemically exposed humans

showed that a range of antibody and cytokine responses associated with both resistance and susceptibility could be elicited, however only those elicited by GST and Sm37-SG3PDH were consistently associated with protective immunity both before and after clearance of infection by praziquantel (Al-Sherbiny *et al.* 2003). Notably however, whilst the latter study noted variation according to host age, the effects of this variation on the protective immune responses identified were not reported or accounted for in statistical analyses (Al-Sherbiny *et al.* 2003). Thus the comparison of resistant (mainly adults) with susceptible (mainly young children) groups does not account for variation in their exposure, an essential consideration (discussed in 1.2.4).

1.8.2 Glutathionine-S-transferase

Of the initial antigens identified GST has emerged as the 'leading vaccine candidate' for schistosomiasis (Capron *et al.* 2002). In *S. mansoni* GST is expressed on the tegument and sub-tegument of adult worms (Taylor *et al.* 1988) and schistosomula (Balloul *et al.* 1985). Its enzymic activity is also required for synthesis of prostaglandin D_2 (PGD_2), a molecule known to inhibit migration of Langerhan's cells (Angeli *et al.* 2001) and promote immunoregulatory cytokine responses at the site of infection (Ramaswamy *et al.* 2000). A variety of GST preparations have been shown to induce a degree of resistance to *S. mansoni* infection in mice (Boulanger *et al.* 1991; Xu *et al.* 1991; Lebens *et al.* 2003), rats (Grezel *et al.* 1993) and *S. matthei* in cattle (Grzych *et al.* 1998). However, evidence for a reduction in pathology and parasite fecundity in *S. haematobium* infected primates after GST vaccination (Boulanger *et al.* 1995; Boulanger *et al.* 1999) has led to a focus on an ShGST-based vaccine rather than its *S. mansoni* counterpart (Bergquist and Colley 1998; Bergquist *et al.* 2002; Al-Sherbiny *et al.* 2003). It is hypothesised that reducing immunopathology via inducing anti-fecundity immune responses may be more achievable than induction of sterile immunity in endemically-exposed human populations. The ShGST-induced anti-fecundity effect appears to be primarily antibody mediated since vaccination induces strong IgA and IgG responses in mice (Lane *et al.* 1998) and humans (Remoué *et al.* 2000; Capron *et al.* 2002). In *S. mansoni* models IgA and IgE-mediated cell cytotoxicity (Grezel *et al.* 1993) and passive transfer of SmGST-specific neutralising antibodies (Xu *et al.* 1993) can lead to a reduction in egg viability, although the same experiments have not been reported for ShGST.

ShGST remains the only purified schistosome antigen to be used in a Phase II clinical trial and Phase III trials of GST vaccination in combination with praziquantel are on-going (NIH 2009), however much of the raw data obtained from these studies has never been published outside of review articles (Capron *et al.* 2002; Wilson and Coulson 2006). The most recent correlate studies in humans endemically exposed to *S. haematobium* showed gender distinctions in GST-specific serum antibodies (Remoué *et al.* 2000; Remoué *et al.* 2001) and PBMC cytokine production (Remoué *et al.* 2001), but published data was not analysed in the context of resistance to re-infection or reduced excretion of eggs. Furthermore, although initial studies in mice suggest that ShGST vaccination elicits poor T cell effector responses (Lane *et al.* 1998) no studies to-date have investigated the relationship between GST-specific cytokine responses and immune-mediated protection in human schistosomiasis.

In addition to its proposed importance as a vaccine candidate, GST is also an abundantly expressed schistosome antigen with isoforms expressed by larval, adult and egg-stage *S. mansoni* (Balloul *et al.* 1985; Curwen *et al.* 2004) and adult *S. haematobium* (Mutapi *et al.* 2005). Thus, there are several advantages to investigating GST-specific immune responses in conjunction with those directed against crude antigen preparations: a) the large amount of potentially non-protective target antigens in crude parasite preparations may obscure changes in immune responses directed against individual antigens that may promote protective immune responses, such as GST, b) reactivity of serum antibody responses to GST isoforms vary with age and infection intensity in *S. haematobium*-exposed populations and thus may reflect host 'exposure history' (Agnew *et al.* 1996; Mutapi *et al.* 2008) and c) exposure to GST is known to change after treatment in humans (Mutapi *et al.* 2005). Thus, purified GST may be a useful indicator of *S. haematobium*-specific immune responses in humans both before and after treatment.

1.9 Helminth therapy

1.9.1 Helminths and the 'hygiene hypothesis'

In contrast to the concerted efforts to control natural helminth infections in endemic areas, recent interest has developed in how artificial helminth infection may be exploited as an immunotherapy in non-endemic areas. *Schistosoma* spp. parasites are not alone in their

ability to regulate human immune responses and hypo-responsiveness to parasite and bystander antigens is also a common feature of human nematode infections (Yazdanbakhsh *et al.* 2002; Jackson *et al.* 2009). Both the immunoregulatory capacity of parasitic helminths and the geographical segregation of helminth-endemic regions from those where allergy and autoimmune conditions are common has led to speculation that helminthiases reduce the risk of immune-mediated disease (Yazdanbakhsh *et al.* 2002). This hypothesis stems from earlier observations that that exposure to bacterial infections during early life is negatively correlated with atopy (Matricardi *et al.* 2000) and atopy is more common in urban than in rural populations (Yemaneberhan *et al.* 1997; Riedler *et al.* 2000; von Ehrenstein *et al.* 2000). A potentially unifying explanation for these observations comes from the 'hygiene hypothesis', which suggests that the reduced exposure to infections and environmental antigens due to improved sanitation and healthcare in affluent urbanised populations also deprives these populations of helminth-mediated immunomodulation of the immune response to non-pathogenic antigens (e.g. allergens and auto-antigens).

The phenomenon that parasitic helminths can reduce immune-reactivity to allergens was first identified in *S. haematobium*-infected school-children (van den Biggelaar *et al.* 2000). Since then a wealth of studies have investigated the association between immune-mediated disease and helminth infections in humans and recent meta-analyses confirm a consistent association between *Ascaris lumbricoides, Trichuris trichuria*, hookworm and schistosome infections and a reduced risk of skin sensitisation to allergens (Feary *et al.* 2011) and hookworm infections and a reduced risk of developing asthma (Leonardi-Bee *et al.* 2006).

1.9.2 Experimental helminth infections

Recent clinical trials have attempted to evaluate whether experimental helminth infection can ameliorate symptoms of human disease (summarised in Table 1.2). These studies have mainly focused on 2 helminth species, the human hookworm *Necator americanus* and the porcine nematode *Trichuris suis*, although there are also cases of individuals suffering from severe immune-mediated diseases voluntarily infecting themselves (e.g. *T. trichiura* (Broadhurst *et al.* 2010)). These GI species have been favoured over tissue-dwelling helminths such as schistosomes since they have a relatively lower pathogenic potential and more restricted locale within the host. *T. suis* has the added benefit of being incapable of

establishing long-term infections in humans and is readily eliminated after a few weeks of infection (Summers *et al.* 2005c).

Natural *T. suis* infection occurs in pigs via ingestion of eggs, after which larval worms hatch in the gut lumen without migrating through host tissues. The Th2 response elicited by *T. suis* in the porcine host is well characterised and comprises increasing numbers of parasite-specific IL-4-secreting cells with time post-infection (Steenhard *et al.* 2007), mucosal goblet cell hyperplasia (Kringel *et al.* 2006), elevation of blood eosinophils (Kringel *et al.* 2006; Steenhard *et al.* 2007), mast cells (Kringel *et al.* 2006) and basophils (Kringel and Roepstorff 2006). Although chronic human infections with *T. trichiura* are characterised by a mixed profile of parasite-specific cytokines (IFNγ, TNFα, IL-4, IL-9, IL-10 and IL-13) and antibody isotypes (IgG1, IgG4, IgA, and IgE) (Faulkner *et al.* 2002), the immune responses elicited by *T. suis* infection in helminth-naïve human hosts are less well characterised (discussed below).

The hookworm life-cycle differs from that of *T. suis* as infection occurs via percutaneous penetration by infective-stage free-living larvae (L3). Following infection, hookworm larvae migrate through the lungs and into the GI tract, where adult parasites feed on host blood, mate and produce eggs, which are transmitted to the environment in host faeces Although heavy infestations can lead to anaemia and stunted growth (Hotez *et al.* 2004), chronic hookworm infections are usually asymptomatic and are particularly associated with a reduced risk of asthma and atopy (Leonardi-Bee *et al.* 2006; Feary *et al.* 2011) . Hookworms induce a mixed Th1/Th2-type cytokine response during natural human infections, but cytokine production during experimental infections appear to be more Th2 polarised (McSorley and Loukas 2010).

1.9.3 Diseases targeted in clinical trials

1.9.3.1 Inflammatory bowel diseases

Inflammatory bowel disease (IBD) is a collective term for pathologies caused by hyperactive immune responses in the gut. The 2 major forms of IBD are Crohn's disease and ulcerative

Species	*Disease*	*Patients (controls)*	*Study design*	*Treatment*	*Doses*	*Dose interval*	*Clinical effects*	*Side-effects*	*Immune responses*	*Reference*
Necator americanus	Crohn's disease	9, 4*	open label	25-50 L3 larvae	1, 2	27-30 week	reduced DAI	itching, reduced Hb	eosinophilia	Croese *et al*, 2006
	Coeliac disease	10 (10)	double-blinded, placebo-controlled	5-10 L3 larvae	2	12 week	NS	flatulence, nausea, bloating, enteritis	eosinophilia, transient leukocytosis	Daveson *et al*, 2011
	Allergic rhinitis	16 (16)	randomised, double-blinded, placebo-controlled	10 L3 larvae	1		NS	itching, skin redness, abdominal pain, appetite loss	eosinophilia	Feary *et al*, 2009
	None	3	self-treated	25-50 L3 larvae	1		NS	skin rash, enteropathy	eosinophilia	Croese *et al*, 2006
Trichuris suis	Ulcerative colitis	3, 2*	open label	2500 live ova	1, 2	3 week	reduced DAI	none reported	undetermined	Summers *et al*, 2003
	Crohn's disease	4, 2*	open label	2500 live ova	1, 2	3 week	reduced DAI	none reported	undetermined	Summers *et al*, 2003
	Ulcerative colitis	30 (24)	randomised, double-blinded, placebo-controlled	2500 live ova	6	12 week	reduced DAI	none reported	undetermined	Summers *et al*, 2005a
	Crohn's disease	29	open label	2500 live ova	8	3 week	reduced DAI	none reported	undetermined	Summers *et al*, 2005b
	Allergic rhinitis	50 (50)	randomised, double-blinded, placebo-controlled	2500 live ova	8	21 day	NS	flatulence, diarrhoea, GI discomfort+	eosinophilia, *T. suis*-specific Ab	Bager *et al*, 2010
	Multiple sclerosis	5	open label	2500 live ova	6	2 week	fewer new MRI lesions	none reported	serum IL-4 & IL-5, eosinophilia, *T. suis*-specific Ab	Fleming *et al*, 2011
Trichuris trichiura	Ulcerative colitis	1#	self-treated	500, 1000, 2000 germinated eggs	3	3 month, 3 year	reduced DAI	intense chronic infection established	*T. trichuria*-specific IL-4, IFNγ, IL-17 & IL-22, reduced neutrophil infiltration	Broadhurst *et al*, 2010

Table 1.2. Clinical trials of experimental helminth infection as therapies for immune-mediated disease in humans. *2 participant groups involved in study (group sizes shown). +some patients received treatment for gastrointestinal symptoms, #self-administered periodic doses of 5-aminosalicylates. DAI – Disease activity index (disease and study specific clinical markers), NS – no significant effect, Ab – antibodies, Hb – Haemoglobin

colitis, the former of which develops from Th1 and Th17-type responses, whilst the latter is more often associated with Th2-type responses (Brand 2009). American trials of experimental *T. suis* infection in both ulcerative colitis and Crohn's disease have demonstrated clinical efficacy in the absence of adverse side effects (Summers *et al.* 2003; Summers *et al.* 2005a; Summers *et al.* 2005b). Although a single dose of *T. suis* ova (TSO) was found to alleviate symptoms in initial trials (Summers *et al.* 2003), this effect was short-lived and subsequent larger-scale randomised double-blinded placebo-controlled trials were conducted using repeated doses (Summers *et al.* 2005a; Summers *et al.* 2005b).

Whilst strongly contested (Summers *et al.* 2005c) , concern that repeated doses of TSO may incur a risk of chronic infection and do not induce lasting reductions in IBD symptoms prompted interest in experimental *N. americanus* infections, which have been shown to reduce Crohn's disease activity index for up to 45 weeks after a single dose of infective larvae (Croese *et al.* 2006). However, the latter study was conducted in only a small number of patients and clinical improvements appeared to be delayed by infection-induced eosinophilia (Croese *et al.* 2006).

1.9.3.2 Allergic airway diseases

Allergic airway diseases are attractive targets for helminth therapy since current therapies rely on prolonged treatment regimens and pose a risk of adverse side-effects (Varney *et al.* 1991). Allergic rhinitis, which was the target of TSO therapy in the clinical trial described in chapter 7, is amongst the most common allergic airway diseases and affects approximately 20% of Western Europeans (Varney 1991). Symptoms include sneezing, blocked nose, red/itchy and watery eyes. Where rhinitis results from allergy to pollen, these symptoms are seasonally exacerbated and are particularly debilitating when environmental pollen counts are high. Successful therapies for sensitivity to aero-allergen have been shown to depend on modification of local immune responses in the nasal mucosa (Wachholz *et al.* 2002; Senti *et al.* 2008). Therapeutic GI nematode infections would therefore be required to alter systemic immune responses in order to influence symptoms at sites distal to the gut, as has been observed in helminth-infected mouse models of allergic airway diseases (Wilson *et al.* 2005; Mangan *et al.* 2006).

To date neither hookworm nor TSO therapy has shown significant clinical benefit to allergic airway disease or asthma relative to placebo-treated controls (Feary *et al.* 2009; Bager *et al.* 2010a; Feary *et al.* 2010). Furthermore, both have been associated with a higher incidence of adverse side-effects (Bager *et al.* 2010a; Feary *et al.* 2010), which had not been reported in previous TSO treatment studies. Importantly, the immunological basis for these clinical observations has not been explored.

1.9.3.3 Multiple sclerosis

A small number of studies have investigated the efficacy of helminth therapy in multiple sclerosis (MS) (Fleming *et al.* 2009; Fleming *et al.* 2011), a relapsing-remitting autoimmune disease affecting the central nervous system and perpetuated by pro-inflammatory Th1 and Th17 cells (Juszczak and Glabinski 2009). Repeated doses of TSO were orally administered at 2 week intervals over a 3 month period and, of the 5 enrolled participants, all but one had a reduced number of new MS lesions assessed by Magnetic Resonance Imaging (MRI), although this reduction did not affect neurological symptoms (Fleming *et al.* 2011).

1.9.4 Immune responses during experimental helminth infection

Of the studies investigating immune responses during experimental helminth infection all report a treatment-induced increase in eosinophil counts relative to pre-treatment levels (Wright and Bickle 2005; Croese *et al.* 2006; Blount *et al.* 2009; Feary *et al.* 2009; Bager *et al.* 2010a; Daveson *et al.* 2011; Fleming *et al.* 2011). As would be expected, treatment is also associated with an increase in parasite-specific antibodies, reflecting exposure to helminth antigens in previously helminth naïve individuals (Blount *et al.* 2009; Bager *et al.* 2010a; Fleming *et al.* 2011).

All trials of *N. americanus* have reported adverse side-effects during experimental infection (Croese *et al.* 2006; Feary *et al.* 2009; Feary *et al.* 2010; Daveson *et al.* 2011) which may be attributable to duodenal eosinophilia and allergic reactivity to larvae in the skin. Experimental *N. americanus* infections in individuals with allergic rhinitis has also been shown to elevate PBMC IL-5 and IL-13 responses to Staphyloccocal enterotoxin (peaking within the first 4 weeks of administration of a single dose of 10 larval parasites) relative to placebo-treated controls, although this difference was not statistically significant and had

declined to levels equivalent to placebo-treated participants by week 10 (Blount *et al.* 2009). Interestingly TNFα levels in the same individuals were marginally lower (week 4-12 post infection) and IL-10 marginally higher (week 8-12 post infection) than PBMC from placebo-treated participants after enterotoxin stimulation, though these differences were also not statistically significant (Blount *et al.* 2009).Changes in cytokine responses did not correspond to changes in the total number of circulating T cells, IL-4+ T cells, CD4+CD25+Foxp3+ Tregs or total IgE titres (Blount *et al.* 2009), but suggest that even light, single dose infection with a gastrointestinal helminth can influence the host cytokine environment within weeks of exposure without exacerbating allergen-specific IgE responses (Blount *et al.* 2009). Th2-type cytokine responses (IL-5 and IL-13) to somatic hookworm antigens have also been shown to increase transiently in an individual who self-infected with *N. americanus* larvae and this increase recurred upon a second percutaneous dose of parasites (Wright and Bickle 2005). Investigation of these immunological effects in larger cohorts and over a longer exposure period are warranted to investigate the influence of such immunological changes on allergic disease and the applicability of anecdotal observations.

Only 2 studies to date have investigated cytokine responses during experimental human *Trichuris* spp. infections involving only 5 individuals. For *T. suis* ova treated participants, serum cytokine levels varied considerably between individuals, but a sustained increase in IL-4 and IL-5 and an initial increase in IL-10 and IL-13 (in 4 out of 5 individuals), but not IFNγ and IL-2, was observed in most participants relative to pre-treatment levels (Fleming *et al.* 2011). These changes in serum cytokines did not correspond to variations in the frequency of circulating CD4+CD25+Foxp3+ Treg or monocytes expressing markers of alternative activation (CD14+CD124+CD23+) (Fleming *et al.* 2011). Importantly, unlike hookworm infections which have a lung migratory stage to which transient changes in cytokine responses have been previously attributed (Blount *et al.* 2009), *T. suis* remains in the gut lumen and does not migrate through host tissues. Experimental *T. trichiura* infection lead to an increase in a mixed profile of Th2 (IL-4), Th1 (IFNγ) and Th17 (IL-17) associated cytokines and IL-22 in a single individual who repeatedly self-infected over several years (Broadhurst *et al.* 2010). Gut biopsy analyses showed that infection lead to reduced expression of pro-inflammatory genes in colitic tissue and, whilst IL-17+ CD4+ T cells were prominent during active disease, infiltration by IL-22+ CD4+ T cells were associated with clinical improvements during infection (Broadhurst *et al.* 2010). No corresponding change was observed in the Foxp3+ Treg population during colitis relapse or remission (Broadhurst

et al. 2010). Although conducted in only a single individual and somewhat confounded by self-administered doses of anti-inflammatory drugs, the latter study presents the first insight into human immune responses to experimental infection in local tissues. Larger scale studies are required to investigate the reproducibility of these promising observations.

Since helminth therapy is proposed to act via modulating the host immune response to allergens and auto-antigens it is noteworthy that despite marked changes in parasite-specific immune responses, experimental *T. suis* infection was not found to alter allergen-specific antibody responses during the clinical trial in allergic rhinitis sufferers described in chapter 7 (Bager *et al.* 2010a). Similarly, experimental hookworm infection failed to alter the number of IFNγ + T cells responding to dietary gluten allergens in patients with Coeliac disease (Daveson *et al.* 2011). Both studies found no clinical efficacy relative to placebo controls. However, the absence of data on parasite-specific cytokines mean that the clinical read-outs of these trials cannot be related to changes in immune polarisation, which is proposed as the major cause of hypo-responsiveness during chronic helminth infections (Figueiredo *et al.* 2010).

1.10 Thesis outline

This thesis will address some of the key gaps in our current understanding of the immunobiology of human helminth infection, particularly in relation to cytokine responses. The specific aims of each aspect of the study, design and implementation of the collaborative projects of which it is a part and the methods shared between subsequent chapters are outlined in chapter 2.

In chapter 3 I directly compare the innate inflammatory, Th1, Th2 and Th17-type cytokine profile elicited by *S. haematobium* cercariae, adult worm and egg-stage antigens in a population endemically-exposed to infection. This is the first systematic analysis of how this comprehensive range of different cytokine responses may differ according to parasite life-cycle stage.

In chapter 4 I investigate the complex relationship between host age, current infection levels and *S. haematobium*-specific cytokine responses and how this relationship may contribute to epidemiological patterns of natural schistosome infection. This study is also the first to investigate human *S. haematobium* GST-specific cytokine profiles in the context of variation in host age and infection intensity.

The focus of chapter 5 is how praziquantel treatment alters *S. haematobium*-specific cytokine responses, including analysis of changes in individual cytokine responses and how combined cytokine responses shift relative to pre-treatment patterns.

Chapter 6 provides a further investigation of how praziquantel treatment influences the *S. haematobium*-specific cytokine environment by first determining how pre-treatment infection intensity affects the magnitude of change in cytokine responses after treatment and going on to show that post-treatment cytokine profiles may influence the risk of subsequent re-infection.

Whilst chapters 3-6 investigate helminth-specific cytokine responses during natural exposure to infection in an endemic setting, chapter 7 characterises systemic and parasite-specific cytokine responses during experimental *Trichuris suis* infection in a cohort of helminth-

naïve allergic rhinitis sufferers. Importantly this study is the first to investigate how experimental helminth infection affects allergen-specific cytokine responses, a key target for allergy immunotherapies.

Chapter 8 provides a general discussion of my findings, how these relate to the existing literature and proposals for future research in the field.

Chapter 2

Aims, study design and methods

2.1 Introduction

This study comprises 2 distinct projects, the first being part of a large-scale immunoepidemiological survey of *Schistosoma haematobium* infection in an endemically-exposed community in Zimbabwe and the second being conducted as part of a clinical trial of experimental *Trichuris suis* infection as an immunotherapy for grass pollen allergy (allergic rhinitis) in a cohort of Danish volunteers. The unifying theme of both studies is the characterisation of how helminth parasites affect host cytokine responses in the context of variation in the host, parasite and their shared environment.

To date studies of helminth-specific cytokine responses in both animal models and humans have focused on a small number of 'characteristic' cytokines, particularly those associated with Th1 and Th2-type responses (reviewed by Allen and colleagues (Allen and Maizels 1997; Diaz and Allen 2007; Allen and Maizels 2011)), as a means of identifying immune phenotype. However, analyses of cytokine responses assayed in field studies of helminth immunobiology in humans have tended to focus on cytokines as individual responses, rather than the broader immune 'phenotype' from which they are derived (chapter 1.5). Importantly effector immune responses are perpetuated by multiple co-incident cytokines derived from a variety of cell types and thus individual cytokine dynamics never occur in isolation. Whilst there is clearly an argument for selection of cytokines according to *a priori* hypotheses, effective cytokine multiplex assays, current statistical tools and our increased understanding of the 'cytokine environment' mean that assaying a wider range of biomarkers does not preclude hypothesis-driven analysis. Furthermore, inconsistencies in assay protocols and heterogeneity in human populations have inevitably led to variation in the findings of different studies supporting a cohesive approach to within-study cytokine analysis in addition to between-study comparisons (reviewed by(Bourke *et al.* 2011)).

The purpose of the current study is to characterise how cytokine profiles, in addition to levels of individual cytokines, relate to helminth immunobiology during both natural and experimental infections. This approach warranted both assessment of a broader range of cytokines than that assayed by previous studies and use of multivariate statistical methods adapted from population ecology (McCune and Grace 2002) and psychometrics (Rummel 1970) that allowed cytokine responses to be analysed in combination.

This chapter describes the specific aims and study design of each aspect of the study and their shared practical and statistical methods. For the *S. haematobium* study, which addressed different research questions using data collected from the same population (chapter 3-6), the participant selection criteria and epidemiological features of the study cohort are also outlined.

2.2 Study aims

As part of the immunoepidemiological survey of *S. haematobium* infection, the specific aims of my study were to:

- Compare the cytokine environment elicited by *S. haematobium* cercariae, adult worm and egg antigens in peripheral whole blood samples collected from a cohort endemically exposed to infection (chapter 3)
- Determine the relationship between *S. haematobium* infection intensity, age and *S. haematobium*-specific whole blood cytokine responses and how these relationships may correspond to epidemiological patterns of infection (chapter 4)
- Characterise changes in *S. haematobium*-specific whole blood cytokine responses 6 weeks after anti-helminthic treatment (chapter 5)
- Relate treatment-induced changes in *S. haematobium*-specific cytokine responses to infection levels prior to treatment and the risk of re-infection within 18 months of treatment (chapter 6)
- Characterise the pre and post-treatment whole blood cytokine responses elicited by the *S. haematobium* vaccine candidate antigen glutathionine-S-transferase 28kDa (GST) (chapter 4, 5 and 6)

Within the clinical trial of experimental *T. suis* infection in patients with grass pollen allergy, my specific aims were to:

- Characterise changes in the systemic cytokine environment over the course of the grass pollen season in plasma samples from *T. suis* and placebo-treated patients (chapter 7)
- Investigate the effects of *T. suis* infection on allergen and parasite-specific peripheral blood mononuclear cell (PBMC) cytokine responses (chapter 7)

2.3 Immunoepidemiological survey of *S. haematobium*-specific cytokine responses in an endemically-exposed population

The following study design and methods are shared between chapters 3-6.

2.3.1 Study design

This aspect of the study is part of a 3 year longitudinal treatment-re-infection survey of *S. haematobium* immunoepidemiology being conducted by researchers at the University of Edinburgh, University of Zimbabwe (UZ) and the National Institute of Health Research (NIHR), Harare. The project was made up of both a cross-sectional survey of schistosome infection and immune responses prior to treatment and a longitudinal follow-up survey of re-infection levels and immune responses in a sub-set of participants 6 weeks, 6 months and 18 months after praziquantel treatment. The focus of the current study is the *S. haematobium*-specific cytokine response elicited in peripheral whole blood samples collected before and 6 weeks after treatment.

The cross-sectional survey involved mass, school-based recruitment of school children (Figure 2.1A) and members of the local community over a 3 week period. School-based recruitment facilitated efficient recruitment and follow-up of compliant participants and dissemination of information about the study to the local community. A population census was conducted during the recruitment phase. All compliant participants provided stool and urine samples for parasitological analysis (Figure 2.1B and C) and a sample of venous blood was collected for immunological assays (Figure 2.1D). Samples were transported from the study sites for processing in the diagnostics laboratory of the local hospital on the day of collection. Samples for subsequent immunological analysis were frozen and transported to the University of Edinburgh.

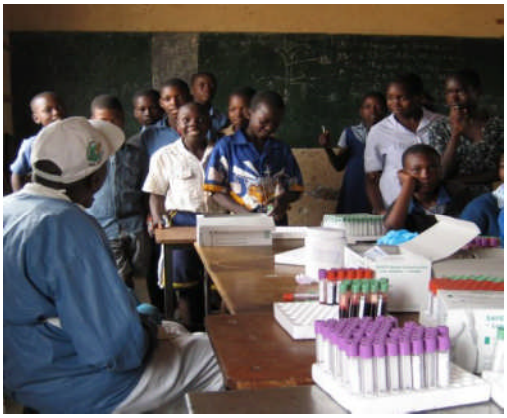
After collection of cross-sectional samples, all compliant individuals were treated with a single standard dose of praziquantel (40mg/kg body weight) and albendazole (400mg) as

A.

B.

C.

D.



E.

Figure 2.1. Photographs from immunoepidemiological field survey of Magaya community, Zimbabwe. A) Recruitment of participants at Magaya primary school, B) Collection of stool and urine samples for parasitological analysis, C) Filtration of urine samples to quantify *S. haematobium* infection intensity (egg counts/10ml), D) Collection of serological samples and E) Praziquantel treatment (40mg/kg).

recommended by the W.H.O. (Montresor *et al.* 1998) (Figure 2.1E). Both praziquantel and albendazole were administered irrespective of infection status in accordance with ethical permissions obtained for the study (see 2.3.2). Repeat doses of anti-helminthic were provided to individuals found to be persistently excreting schistosome or soil-transmitted helminth (STH) eggs, but these individuals were not included in post-treatment analyses. Follow-up samples were collected 6 weeks, 6 months and 18 months post-treatment as part of an on-going survey of re-infection post-treatment within the population.

2.3.2 Study site and participants

The study was conducted in Murehwa district in Northern Zimbabwe. The study area experiences a dry season from May to October with low precipitation, low humidity and high average temperatures and a rainy season from November to April when temperatures are cooler. The local climate means that transmission of *Plasmodium* spp. parasites is seasonal, and this has been confirmed by collation of district level data on the number of malaria cases in children collected over a 10 year period (Mabaso *et al.* 2005). Country-wide *Plasmodium* spp. infections peak during the rainy season from February to May (Taylor and Mutambu 1986; Mabaso *et al.* 2005) and the predominant species is *Plasmodium falciparum,* however infection prevalence is low, particularly in high-altitude areas such as our study site (Taylor and Mutambu 1986). The pre-treatment survey of Magaya community was initiated in September 2008 and the 6 weeks post-treatment follow-up survey was conducted in late October of the same year.

Samples were collected from 5 schools in 3 distinct locations within Murehwa district; Magaya primary and secondary schools, Chipinda primary and secondary schools and Chitate primary school. These schools were selected from a pre-survey of schools in the area for the presence of *S. haematobium* infection and the relatively low prevalence of *S. mansoni* and STH infections. The schools are non-residential and enrolled students travel from a range of local villages and homesteads. All 5 schools are located in the rural surrounds of Murehwa, the largest town in the district. Adult members of the local community, including school teachers, parents, relatives and friends of children enrolled at the schools, were informed about the study and invited to participate through community meetings held in during the pre-survey period and were enrolled at school sites. The cohort recruited from Magaya community is the main focus of my study (Chapter 3, 4, 5 and 6) as it was found to

have the highest prevalence of *S. haematobium* infection of the 3 communities. In total, demographic data was collected from 675 members of Magaya community prior to treatment, of which 423 were primary school children, 158 were secondary school children, 94 were community members who did not attend school. Of these participants, 35 family groups (mean family group size identified: 2.5 members, range: 2-5 members) were identified amounting to a total of 88 participants. 13 recruited adult community members were directly related (father, mother or grandparent) to school-aged children in the cohort. Although familial groups were identified, only a minority of recruited adults were directly related to school-age recruits, indicating that school-based recruitment did not bias the adult cohort that enrolled in the study.

The schools and a subset of water contact sites and participant's homesteads in the area were surveyed and mapped using hand-held global positioning systems (GPS) by my colleagues Kate Mitchell and Laura Appleby. Water contact sites ranged from dammed pools, rivers, some of which are seasonal, and wells. The majority of study participants relied on natural water sources for at least some of their domestic activities (assessed by questionnaire, Appendix 1). On average study participants made 2 contacts per day with water sources that posed a potential risk of schistosome infection (i.e. stagnant or slow-moving freshwater sources) (Mitchell and Appleby, un-published data). *S. haematobium* intermediate host snails (*Bulinus* spp.) were also found at water contact sites identified by the GPS mapping survey. Although it would have been desirable to conduct an observational water contact survey, as has been conducted previously (Chandiwana and Woolhouse 1991), the extensive range and distribution of water contact sites meant that this approach was not practically feasible. A GPS map of the study area produced by Kate Mitchell and Laura Appleby is shown in Figure 2.2.

2.3.4 Ethical considerations

Ethical approval to conduct the study was granted by the Medical Research Council of Zimbabwe (MCRZ) and institutional approval was granted by the Institute Review Board of the University of Zimbabwe (UZIRB). Permission to recruit participants at the study sites was provided by the Provincial Medical Director.

Only compliant participants were included in the study and all recruited participants were

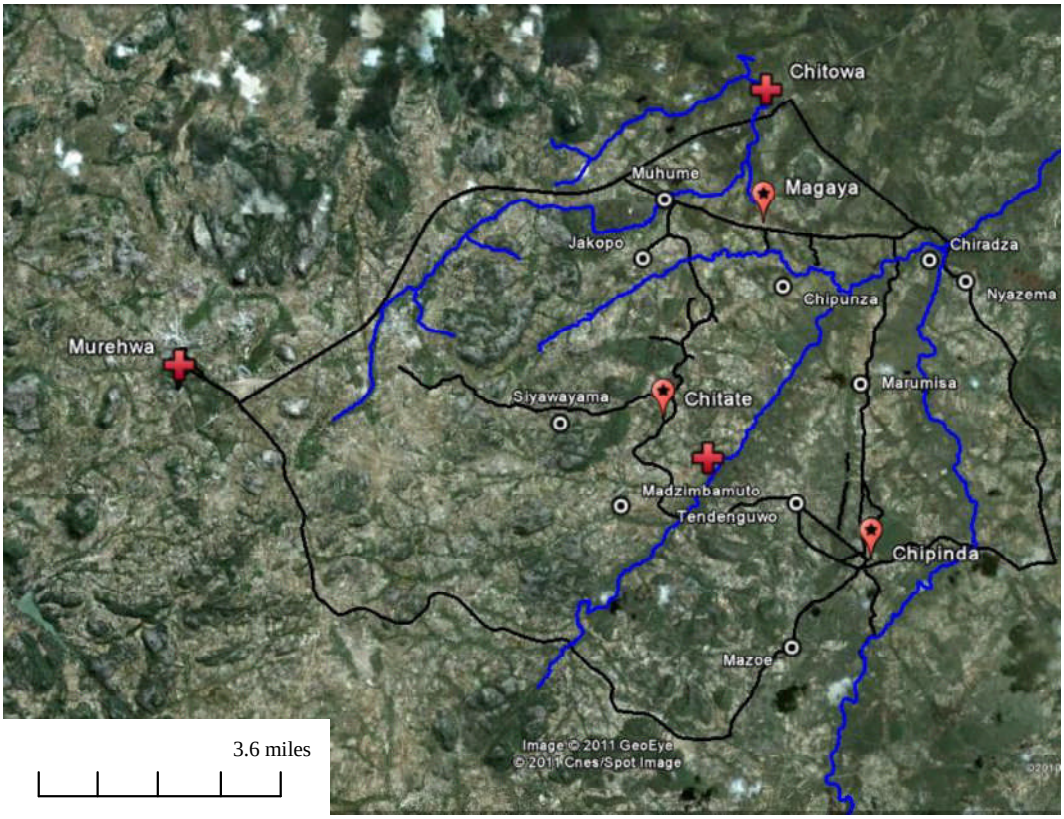


Figure 2.2. Map of the field study area. The map was generated from GPS co-ordinate data collected on-foot and co-ordinates were overlaid onto a satellite image of the area obtained from GoogleEarth (Mitchell and Appleby, un-published data). Markers indicate school sites where samples were collected (red markers), village centres and participant homesteads (white circles), rivers (blue lines), roads (black lines) and local clinics/hospitals (red crosses). Water contact sites were distributed throughout the area. Scale is in miles (mi) and map co-ordinates are: 17°39°'57.29" South, 31°52°'24.08" East. Elevation: 4489ft.

free to withdraw at any time. All participants were given a full explanation of the study aims, approach and procedures in the local language (Shona) after which they were invited to participate in the study. Enrolment involved provision of informed written consent, which was recorded along with a participant's name and age prior to sample collection. A parent or guardian provided consent for children aged less than 18 years at recruitment. Each participant was allocated a unique case number and all pre and post-treatment samples and data collected from an individual was identified by case number. Collection of blood samples and administration of praziquantel and albendazole was carried out by nursing staff from Murehwa District Hospital and local clinics.

2.3.5 Methods

2.3.5.1 Population survey

All participants completed a questionnaire (Appendix 1)to assess name, age, date of birth, sex, relatives within the cohort, place of residence, residential history, typical water contact behaviour (on day of survey and over the preceding week), prior knowledge of schistosome infection and treatment history for schistosomiasis (treatment status, type of treatment, date and location of treatment). Questionnaires were prepared in English, but administered to all participants by a native Shona speaker to avoid language, accent or literacy barriers (Mduluza *et al.* 2007). A parent or guardian of children under 5 years of age completed specially-prepared questionnaires on their behalf and a post-treatment follow-up questionnaire to assess any side-effects of treatment (Mutapi *et al.* 2011c). To ensure the accuracy of age and gender data, information from pre-treatment questionnaires was used in concert with school registers and surveys conducted at follow-up visits.

2.3.5.2 Diagnosis & quantification of infection

S. haematobium, *S. mansoni*, STH, *Plasmodium* spp. and HIV infection were diagnosed and quantified in collected samples by experienced technical staff from the NIHR.

2.3.5.2.1 *S. haematobium*

Individuals provided urine samples (between 2 and 6 samples on consecutive days) and were classified as positive for *S. haematobium* infection if eggs were detected in any of the samples that they provided. Eggs were identified on the day of sample collection by filtration of 10ml urine through a nitrocellulose filter (Figure 1B), which was then examined under a light microscope according to Mott's method (Mott 1983). Arithmetic mean egg counts were used in all analyses and participants who provided fewer than 2 urine samples for quantification of *S. haematobium* infection were excluded from the study.

2.3.5.2.2 *S. mansoni* and Soil-transmitted helminths (STH)

S. mansoni and STH infection were assessed using the Kato-Katz technique (Katz *et al.* 1972). Stool samples were first sieved to remove large particles and a minimum of 2 slides per sample were prepared using standard 41.7mg templates. Samples were stained with glycerol-malachite green, which stains stool components but cannot penetrate live helminth eggs making them easier to identify. Eggs were counted under a light microscope and all slides were analysed on the day of collection. As recommended by previous studies a minimum of 2 stool samples with 2 smears per stool were analysed to ensure sensitivity of egg detection (Ebrahim *et al.* 1997).

2.3.5.2.3 *Plasmodium* spp.

Thick and thin blood smear slides were prepared for each participant from freshly collected venous blood, dried at room temperature and fixed with methanol. Malaria infection was detected by microscopic analysis of thick smear blood slides and, if an individual was found to be positive, infection was quantified by counting infected red blood cells on a corresponding thin-smear blood slide. In addition, Paracheck Pf® kits (Orchid, Catalogue# 30302100), a malaria rapid diagnostic test specific for the histidine rich protein 2 of *P. falciparum*, confirmed malaria positive/negative cases.

2.3.5.2.4 Human Immunodeficiency Virus (HIV)

HIV infection was diagnosed using DoubleCheckGold HIV 1&2 Whole Blood test (Orgenics, Catalogue# 70633020), an immunochromatographic assay for HIV-1 and HIV-2-specific antibody using 2-3 drops of fresh whole blood. The test detects circulating

antibodies specific for the immunodominant epitopes of HIV-1 and 2 envelope and gag proteins. All positive cases were confirmed by subsequent enzyme-linked immunosorbent assay (ELISA) analysis detecting sero-reactivity to HIV proteins at the NIHR.

2.3.5.3 Serology

Each individual provided up to 50ml of whole blood (depending on body weight), which was collected via venous puncture into 5 separate collection tubes. 3x 10ml samples were collected into heparinised tubes for isolation of PBMCs and plasma samples. 10ml were collected into a silicon-coated tube without anti-coagulant for isolation of sera. 10ml were collected into a K_2EDTA-treated collection tube, which was subsequently used for whole blood re-stimulation cultures (5ml), HIV diagnosis (2-3drops), preparation of differential cell count slides (200µl), inclusion in a whole blood sample archive (500µl) and plasma (1ml) for subsequent assays of cytokine production. K_2EDTA was chosen for sample collection for whole blood culture because it is known to prevent coagulation of blood cells and also yield viable cells for re-stimulation and cytokine analysis (Mayringer *et al.* 2000; Remick *et al.* 2000). All tubes were inverted after sample collection to ensure mixing of the anti-coagulant with the blood, transported from the field site in chilled containers and processed at the diagnostics laboratory at Murehwa District Hospital.

2.3.5.4 Whole blood re-stimulation culture

2.3.5.4.1 Antigens

Crude homogenates of *S. haematobium* cercariae (cercarial antigen preparation (CAP)), adult worms (whole worm homogenate (WWH)) and eggs (soluble egg antigen (SEA)) were obtained from the Schistosome Biological Supply Centre at the Theodor Bilharz Institute, Giza, Egypt. The centre routinely produces schistosome antigens from an Egyptian strain of *S. haematobium* using established protocols similar to those for preparation of *S. mansoni* antigens (Jassim *et al.* 1987). Freeze-dried antigen preparations were reconstituted in 500µl double-distilled water and protein content was quantified by Bradford assay. All antigens were stored at -20°C in concentrations >1mg/ml. Identically prepared antigens from this parasite strain have been used in previous studies by our research group (Mutapi *et al.* 2008; Reilly *et al.* 2008; Milner *et al.* 2010; Imai *et al.* 2011).

Recombinant glutathionine-S-transferase 28kDa (GST) of a Senegalese strain of *S. haematobium* was provided by Professor Francois Trottein of the Centre of Immunology and Parasite Biology at the Institut Pasteur de Lille, France. The protein was cloned and purified according to previously published protocols (Trottein *et al.* 1992b). In brief, the GST was sub-cloned into a pET-24d(+) vector and expressed in *E. coli* BL21(DE3) cells. The expression product was purified on a sepharose column, dialysed and concentrated to yield an endotoxin-free recombinant protein (Prof. Francois Trottein, Institut Pasteur de Lille, France, personal communication).

Escherichia. coli Maltose-binding protein (MBP) and phytohaemagglutinin (PHA), an activator of T cell proliferation, were used to confirm the viability of cultured cells and their capacity to produce cytokine in response to non-schistosome antigens. MBP was obtained from New England Biolabs and PHA was obtained from Sigma Aldrich.

To determine whether crude antigens and GST were contaminated by endotoxin, preparations of all antigens were cultured overnight with murine bone marrow-derived dendritic cells (BMDC), which are highly sensitive to endotoxin. Parallel cultures were conducted with and without polymixin B (an endotoxin-neutralising antibiotic) according to a previously published protocol (Jenkins *et al.* 2005a). Inflammatory cytokine secretion was assayed by ELISA and indicated no significant difference between levels of TNFα, IL-6, IL-12p40 or IL-12p70 in the endotoxin blocked and un-blocked cultures. Cytokine responses to titrated endotoxin verified the efficacy of polymixin B treatment. From these assays it can be concluded that, if endotoxin is present in the preparations, it not in sufficient quantities to elicit significant levels of inflammatory cytokines from endotoxin-responsive cells *in vitro*. I used this method rather than a quantitative assay of endotoxin (e.g. Limulus ameobocyte assay) because quantitative assays do not provide information on the effect that the level of endotoxin has on *in vitro* cytokine responses, which are the focus of my study.

2.3.5.4.2 Culture conditions

Whole blood cultures were carried out in the diagnostics laboratory of Murehwa District Hospital for a sub-set of samples collected from Magaya community using established culture protocols (Remick *et al.* 2000; Joseph *et al.* 2004a; Walter *et al.* 2006; Mutapi *et al.* 2007b). All antigens were diluted in culture medium (RPMI supplemented with 5U/ml

penicillin, 50µg/ml streptomycin and 1%v/v L-glutamine) to a give a final culture concentration of 10µg/ml (CAP, SEA, WWH and PHA) or 2µg/ml (GST and MBP) after addition of diluted blood (see below). Antigens were plated in duplicate at 500µl/well in 48 well culture plates. 500µl/well of culture media without antigen acted as an un-stimulated control. The same culture protocol has been used in previous studies (Scott *et al.* 2000; Joseph *et al.* 2004a; Joseph *et al.* 2004b; Mutapi *et al.* 2007b).

On arrival at the laboratory 5ml of whole blood from the K_2EDTA-treated collection tube was diluted to 1:3 in 10ml culture medium. 500µl/well of diluted blood was added to antigen-coated wells to give a final dilution of blood of 1:6 and a final culture volume/well of 1ml. Duplicate cultures for each antigen-stimulation were prepared in adjacent wells and all antigen stimulations and control cultures were conducted on the same plate for each individual to ensure identical culture conditions. Where less than 5ml of blood was collected (e.g. due to participant discomfort during sampling) blood was diluted 1:3 in a lower volume and the maximum number of duplicate cultures possible were conducted prioritising SEA, WWH, GST, un-stimulated and agonist control cultures since a limited amount of CAP was available. For this reason, the number of participant whole blood samples stimulated with CAP during the pre-treatment cross-sectional survey (n = 80) was less than for the other antigens (n = 259).

Plates were cultured for 48 hours at 37°C. Whilst a range of culture durations have been used in previous studies of schistosomiasis, and some studies have used separate timepoints for different cytokines (Joseph *et al.* 2004a) a single 48 hour timepoint was chosen for this study according to protocols optimised for assaying production of multiple cytokines (Scott *et al.* 2000; Joseph *et al.* 2004a). To maintain a constant carbon dioxide concentration, all plates were cultured using the OXOID Anaerogen™ Compact anaerobic atmosphere generation system (OXOID, Product code: AN0010 & AN0020). This involved culturing plates in sealed pouches with a sachet of ascorbic acid and activated carbon that reacts with oxygen inside the pouch to reduce atmospheric oxygen to less than 1% and generate stable levels of CO_2 within 30 minutes of activation.

After 48 hours culture supernatants were harvested into individual microtubes and frozen at -20°C. After transport to the University of Edinburgh, samples were stored at -80°C and processed within 18 months of collection.

2.3.5.5 Immunological assays

2.3.5.5.1 Choice of cytokine assays

I chose to assay a panel of 13 cytokines (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-21 and IL-23) associated with established cellular phenotypes from pathway immunology (Diaz and Allen 2007) as a means of comparing stage-specific variations both within and between these immunological paradigms. The large panel of cytokines was chosen to avoid reliance on the Th1/Th2 dichotomy in isolation (Allen and Maizels 1997) and to address the paucity of data on cytokines associated with innate and Th17-type responses in human helminth studies. For interpretation of the cytokine assays I have used innate and CD4+ T cell-based terminology despite the range of cytokine-producing cell types present in whole blood because this terminology is frequently used to describe cytokine responses in studies of human helminthiases and studies of murine and human immunology suggest that these immunological groupings can be used to broadly categorise host immune phenotypes in infection (Diaz and Allen 2007).

To avoid biasing my interpretations according to individual empirical studies, review articles or murine cytokine literature, I conducted a literature search of recent studies on human cytokine responses and the immune phenotype with which they are most frequently associated. The search was not restricted to studies of helminth infection. The keywords: 'human' AND 'cytokine' AND 'Th1'or'Th2'or 'Th17' or 'innate' AND 'inflammatory' were entered into the online PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) and the top 50 hits arising from each combination of keywords were reviewed. The top 100 hits for 'innate' AND 'inflammatory' were reviewed due to the abundance of literature identified as using these terms (n > 64,000). Articles were included in subsequent assessment if they were: 1) published after January 2010, 2) included analysis of IFNγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (all subtypes), IL-13, IL-17 (all subtypes), IL-21 and/or IL-23 activity or production in human cell culture, cell lines, tissues or peripheral blood samples, 3) used the 'innate inflammatory', 'Th1', 'Th2' or 'Th17' terminology in reference to cytokine responses and 4) presented original empirical data (i.e. review papers were excluded). Articles identifying cytokine mRNA, gene sequencing data or intracellular cytokine expression alone were excluded since all cytokines assayed in the present study were detected in cell-free supernatants. 127 of 250 articles reviewed met the inclusion criteria. From this analysis TNFα, IL-6 and IL-8 were grouped as 'innate inflammatory', IFNγ, IL-2

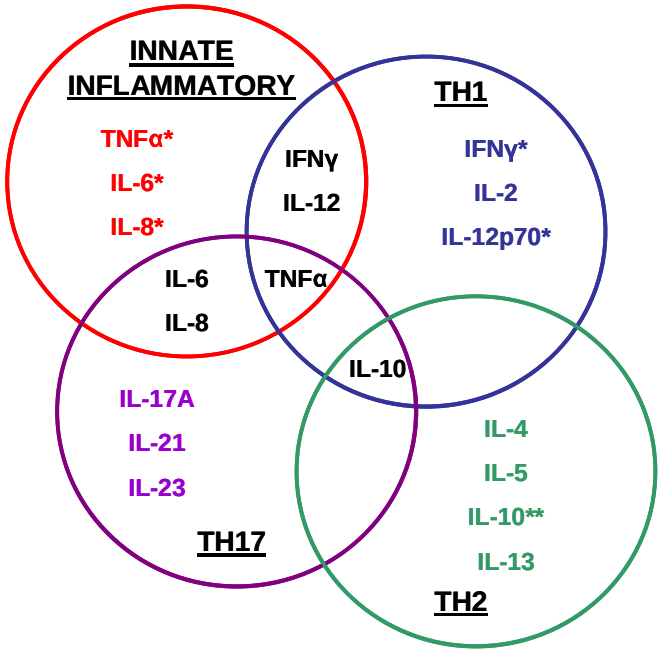


Figure 2.3. Cytokine responses grouped according to their associations with different cellular immune phenotypes. Results of a literature review of empirical human cytokine studies (127 articles) to determine appropriate groupings for assayed cytokines. Keywords ('human', 'cytokine' and 'Th1', 'Th2', 'Th17' or 'innate' and 'inflammatory') were entered into the PubMed online database and the frequency with which a particular cytokine was associated with each phenotype (Th1, Th2, Th17 or innate inflammatory) used to determine its appropriate group. Each circle represents a cellular phenotype and cytokines are included in the phenotype with which they were most frequently associated. Cytokines which were associated with other phenotypes at a lower frequency are given in overlapping areas of the diagram. *also associated with a 'pro-inflammatory' immune phenotype, **also associated with a 'regulatory' immune phenotype.

and IL-12p70 as 'Th1', IL-4, IL-5, IL-10 and IL-13 as 'Th2' and IL-17A, IL-21 and IL-23 as 'Th17'according to the frequency with which they were categorised using these terms (cytokines are also grouped in this way in chapter 1.5.1). The phenotypic groupings of cytokines identified from the literature review are summarised in Figure 2.3. It is of note that many papers also described IL-10 as 'regulatory' or 'anti-inflammatory' (Coelho dos Santos *et al.* 2010; Dorresteijn *et al.* 2010; O'Leary *et al.* 2010; Osakwe *et al.* 2010; van der Does *et al.* 2010) and IL-10 was more often described in these terms than as a Th2-type cytokine. Furthermore, TNFα, IL-6, IL-8, IFNγ and IL-12 were described as being 'pro-inflammatory'(Corrigan and Rowe 2010; Fedele *et al.* 2010; Gruaz *et al.* 2010; Huang *et al.* 2010; Marr *et al.* 2010) and IL-2 as mediating expansion of T cells of a variety of lineages (Pahwa *et al.* 2010; Rivino *et al.* 2010), reflecting that the functions of these cytokines are associated with, but not restricted to these phenotypic groups.

2.3.5.5.2 Optimising ELISA protocol

A cytokine sandwich ELISA technique was used to assay all 13 cytokines from 48 hour whole blood cultures conducted in the field. The ELISA method used was adapted from commercial protocols provided by BD Biosciences, R&D Systems and eBiosciences. To determine optimal assay conditions several preliminary assays were conducted. Firstly, ELISAs were conducted on all recombinant cytokine standard proteins titrated in doubling dilutions from the maximum concentration recommended by the manufacturers to identify the optimum top standard concentration for standard curve generation. ELISA capture and detection antibodies were also titrated to determine respective working concentrations for maximum sensitivity to sample analytes and minimal non-specific binding of detection reagents. Preliminary assays of field samples indicated that diluting samples 1:2 (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, IL-21 and IL-23) and 1:4 (IL-6 and IL-8) was the most appropriate means of capturing the range of cytokine concentrations produced in the whole blood cultures. ELISAs were also conducted using coating buffers, blocking buffers, wash buffers and tetramethylbenzidine dihydrochloride (TMB) substrate reagents provided commercially and equivalent reagents prepared in-house. No notable differences were observed between assays.

During preliminary assays IL-33 was also included in the cytokine panel, however the IL-33 assay used (R&D, Catalogue number: DY3625) was found to be both highly sensitive to variation in ELISA reagents and unsuitable for long-term storage at -80°C at the time. In the

absence of alternative human IL-33 ELISA reagents suitable for large-scale analyses, IL-33 was subsequently omitted from the cytokine assay panel. For the remaining 13 cytokine assays commercial capture and detection antibodies and recombinant standards were used in conjunction with in-house prepared buffers and detection reagents. Antibodies were stored at 4°C and standards aliquoted and stored at -80°C for the duration of the study, according to the manufacturer's recommendations.

2.3.5.5.3 Pre and Post-treatment cytokine ELISA

IFNγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL17A, IL-21 and IL23p19, were assayed in supernatants harvested from 48 hour cultures of whole blood from each individual. All cytokines were assayed for each individual in a single set of assays conducted over 3 days. Supernatants were defrosted overnight at 4°C prior to use. Full details of the specific recombinant standard proteins, capture and detection antibodies used for each cytokine ELISA are given in Table 2.1.

96 well maxisorp ELISA plates (NUNC, Catalogue#DIS-971-010P) were coated with 50µl/well capture antibody diluted in heat-sterilised phosphate-buffered saline (PBS) at 1µg/ml (IFNγ, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A and IL-21) and 2µg/ml (TNFα and IL-23). Plates were stored overnight at 4°C.

The following day plates were washed 3 times in ELISA wash buffer (PBS, 0.05% Tween 20) before incubation for 2 hours at room temperature with 200µl/well blocking buffer (PBS/2% bovine serum albumen (BSA)). Protease-free BSA was supplied by Alpha Diagnostic International (Catalogue#80400).

After blocking, 11 doubling dilutions of 50µl/well recombinant standard cytokine were prepared in PBS/2%BSA in duplicate across the plate with a starting concentration of 1ng/ml (IL-17A), 2ng/ml (IL-23p19 and IL-4), 5ng/ml (IFNγ, TNFα, IL-5, IL-6, IL-8 and IL-10), 10ng/ml (IL-13), 20ng/ml (IL-2 and IL-12p70) and 40ng/ml (IL-21). 50µl/well of PBS/2% BSA without antigen was added to a twelfth set of duplicate wells to act as a 0ng/ml standard (blank). Supernatant samples were diluted 1:2 (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, IL-21 and IL-23p19) or 1:4 (IL-6 and IL-8) in PBS/2% BSA on assay

Analyte	*Reagent*	*Description*	*Isotype*	*Clone*	*Catalogue#*
IFNγ	Capture*	Purified mouse anti-human IFNγ	IgG1,κ	NIB42	551221
	Standard*	Recombinant human IFNγ	-	-	554616
	Detection*	Biotin mouse anti-human IFNγ	IgG1,κ	4S.B3	554550
TNFα	Capture*	Purified mouse anti-human TNFα	IgG1	MAb1	551220
	Standard*	Recombinant human TNFα	-	-	554618
	Detection*	Biotin mouse anti-human TNFα	IgG1,κ	MAb11	554511
IL-2	Capture*	Purified mouse anti-human IL-2	IgG1,κ	5344.111	555051
	Standard*	Recombinant human IL-2	-	-	554603
	Detection*	Biotin mouse anti-human IL-2	IgG1,κ	B33-2	555040
IL-4	Capture*	Purified mouse anti-human IL-4	IgG1,κ	8D4-8	556917
	Standard*	Recombinant human IL-4	-	-	554605
	Detection*	Biotin rat anti-human IL-4	IgG1	MP4-25D2	554483
IL-5	Capture*	Purified rat anti-human IL-5	IgG2a	JES1-39D10	554487
	Standard*	Recombinant human IL-5	-	-	554606
	Detection*	Biotin rat anti-human IL-5	IgG2a	JES1-5A10	554491
IL-6	Capture*	Purified rat anti-human IL-6	IgG1	MQ2-13A5	554543
	Standard*	Recombinant human IL-6	-	-	550071
	Detection*	Biotin rat anti-human IL-6	IgG2a	MQ2-39C3	554546
IL-8	Capture*	Purified mouse anti-human IL-8	IgG2b,κ	G265-5	554716
	Standard*	Recombinant human IL-8	-	-	554609
	Detection*	Biotin mouse anti-human IL-8	IgG2b	G265-8	554718
IL-10	Capture*	Purified rat anti-human and viral IL-10	IgG1	JES3-9D7	554497
	Standard*	Recombinant human IL-10	-	-	554611
	Detection*	Biotin rat anti-human and viral IL-10	IgG2a	JES3-12G8	554499
IL-12p70	Capture*	Purified rat anti-human IL-12p70	IgG1, κ	20C2	555065
	Standard*	Recombinant human Il-12p70	-	-	554613
	Detection*	Biotin mouse anti-human IL-12p40/p70	IgG1	C8.6	554660
IL-13	Capture*	Purified rat anti-human IL-13	IgG1	JES10.5A2	554570
	Standard#	Recombinant human IL-13	-	-	10-1025-C
	Detection*	Biotin mouse anti-human IL-13	IgG1, κ	B69-2	555054
IL-17A	Capture+	Purified mouse anti-human IL-17A	IgG1, κ	eBio64CAP17	14-7178
	Standard+	Recombinant human IL-17A	-	-	14-8179
	Detection+	Biotin mouse anti-human IL-17A	IgG1, κ	eBio64DEC17	13-7179
IL-21	Capture*	Purified mouse anti-human IL-21	IgG1, κ	JI48-1134	558503
	Standard#	Recombinant human IL-21	-	-	10-1666-C
	Detection*	Biotin mouse anti-human IL-21	IgG1, κ	176-539	558502
IL-23p19	Capture+	Purified mouse anti-human IL-23 p19	IgG1, κ	eBio 473p19	14-7238
	Standard+	Recombinant human IL-23	-	-	14-8239
	Detection+	Biotin mouse anti-human IL-12p40/p70	IgG1, κ	C8.6	13-7129

Table 2.1 Cytokine sandwich ELISA reagents. Details of the capture and biotinylated-detection antibodies and recombinant cytokine standard proteins used for both pre and post-treatment assays. The catalogue number, clones and antibody isotype for each product is given according to the product references available at the time of the study. Reagents supplied by: *BDBiosciences, #Insight Biosciences and +eBiosciences.

plates in duplicate wells to a final volume of 50µl/well. Plates were incubated overnight at 4°C.

Plates were washed 3 times before addition of 50ul/well detection antibody at 0.5µg/ml (IFNγ, TNFα, IL-2, IL-5, IL-6, IL-8, IL-12p70 and IL-23) or 1µg/ml (IL-4, IL-10, IL-13, IL-17A and IL-21) in PBS/2% BSA. Assays were incubated for 2 hours at 37°C, after which they were washed 4 times. 50µl/well AMDEX™ streptavidin-horseradish peroxidase (HRP) (GEHealthcare, Catalogue#RPN4401) diluted at 1:6000 in PBS/2% BSA was added and plates were incubated for 2 hours at 37°C.

Excess horseradish peroxidase (HRP)-conjugated streptavidin was removed by washing plates 4 times. The reaction was developed in 50µl/well TMB-based streptavidin-HRP substrate solution, which generates a colourless to blue colour change proportional to the concentration of cytokine in the well. The Tetramethylbenzidine dihydrochloride monohydrate (TMB) substrate solution was prepared fresh for each assay by dissolving 0.024g TMB (Sigma, Catalogue#T8768) in 5ml 1:1 acetic acid and double distilled H_2O and adding it to 145ml of phosphate-citric acid buffer prepared from a 10x stock (25.5g citric acid and 45.7g Na_2HPO_4 in 500ml ultra pure H_2O, adjusted to pH 5). 120µl H_2O_2 was added directly before use. Assays were developed for 1.5min (IL-8) or 5min (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-21 and IL-23) in the dark to reduce the effect of light on the colour-change reaction. The reaction was stopped by adding 50µl/well 25% hydrochloric acid.
Optical densities of each well were read at a wavelength of 450nm using a microplate reader (Emax precision microplate reader, Molecular devices) and mean cytokine concentrations were interpolated from the 12-point recombinant cytokine standard curve using SoftmaxPro spectrophotometer software. All sample cytokine concentrations were multiplied by the appropriate dilution factor.

2.3.6 Cohort selection

After sample collection, cytokine assays and analysis of parasitology and demographic data, the eligibility of each participant for inclusion in each aspect of the study was assessed.

A total of 284 participants provided a whole blood sample at recruitment and of this initial cohort, 129 provided post-treatment follow-up samples at 6 weeks and 53 provided follow-up samples at 18 months post-treatment. Chapters 3, 4, 5 and 6 are based on data collected from relevant sub-cohorts of these individuals. Selection of each cohort was dependent on the research questions being addressed and detailed inclusion/exclusion criteria are described in relevant chapters. An overview of these criteria is given in Figure 2.4.

Since only a limited amount of CAP was available for the study, samples from 80 individuals were randomly selected for culture with CAP. Random allocation was ensured by use of anonymous identification numbers during assays and analysis of demographic and parasitology data only after initial selection of individuals for this aspect of the study.

Selection of sub-cohorts was based on 4 groups of criteria (shown in Figure 4): a) parasitology, b) treatment history, c) residential history and d) cytokine data. Firstly participants were automatically excluded from all aspects of the study if they provided insufficient parasitology data for reliable diagnosis of infections and quantification of schistosomiasis haematobium and mansoni (minimum of 2 urine and 2 stool samples). Furthermore, participants were excluded if they were diagnosed positive for *S. mansoni*, STH or *Plasmodium* spp. infection since immune responses to the antigens of these parasites are known to cross-react with those of *S. haematobium* (chapter 1.4.5). *S. mansoni* prevalence was low in the study cohort and few participants were excluded due to *S. mansoni* co-infection (Figure 2.4). No participants tested positive for STH eggs in stool or *Plasmodium* spp. infection during the study. These findings are consistent with the low prevalence of *S. mansoni* (Midzi *et al.* 2008b; Midzi *et al.* 2010) and sporadic focal distribution of STH (Chandiwana 1989) in Zimbabwe. The absence of malaria-positive cases is most likely due to sampling during the dry season when malaria transmission is low in the study area (Taylor and Mutambu 1986; Mabaso *et al.* 2005). HIV infection is also thought to influence schistosome immunobiology (Karanja *et al.* 1997; Ganley-Leal *et al.* 2006) and, where there were insufficient cases to account for HIV status in statistical analyses, HIV positive cases were also excluded.

The second group of exclusion/inclusion criteria was based on praziquantel treatment. For assessment of pre-treatment cytokine responses to *S. haematobium* antigens (chapters 3 and 4) participants were excluded if they had previously received treatment for schistosomiasis

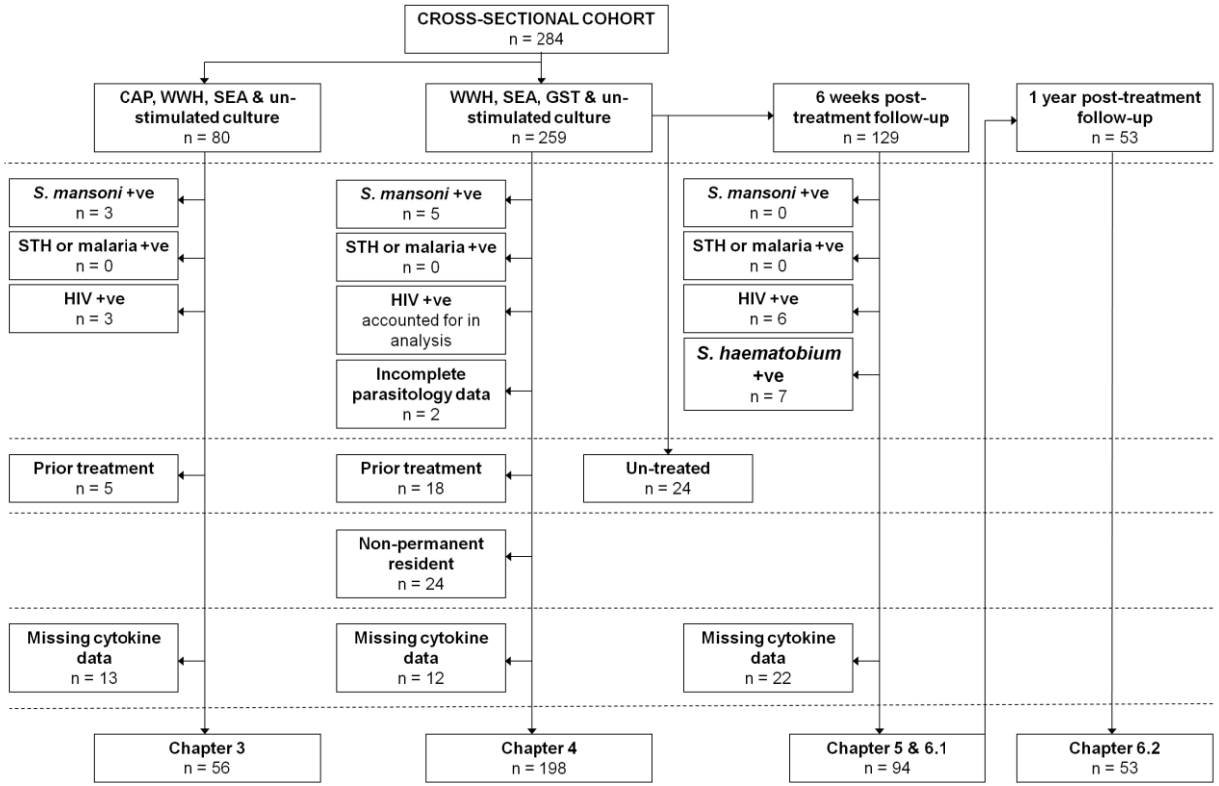


Figure 2.4. Selection of participants for inclusion in each aspect of the *S. haematobium* immunoepidemiological study. Participants who provided blood samples for whole blood culture at recruitment to the cross-sectional survey and subsequent follow-up post-treatment are given at the top of the diagram. Dashed lines indicate groups of inclusion/exclusion criteria (parasitology (top), treatment, residential history and ELISA data (bottom)). The final number of participants selected for each aspect of the study is given at the bottom of the diagram. Boxes indicate number of participants excluded/included and the reason for exclusion in each case.

(assessed by questionnaire). Exclusion on the basis of prior treatment was considered important since studies suggest that treatment can alter acquired immune responses elicited by parasite antigens (chapter 1.7.3). Selection of an area of Zimbabwe that has not been included in national mass anti-helminthic treatment programmes meant that few participants were excluded on the basis of prior treatment. For assessment of post-treatment cytokine responses (chapters 5 and 6) participants who had not received treatment after initial sampling or who had received treatment for schistosomiasis prior to recruitment were excluded.

The third group of exclusion/inclusion criteria was based on residential history. This was considered particularly important for assessment of the relationship between cytokine responses, infection intensity, age and exposure history (chapter 4), since age is only reflective of duration of endemic exposure to *S. haematobium* infection if a participant is a life-long resident of an endemic area. Furthermore, the cytokine responses of individuals with short-term or recent exposure to schistosome infection are known to differ from those of endemically-exposed individuals (Caldas *et al.* 2008). Heterogeneity in the duration of residence in the study site (from 30 years to 6 months) meant that variation due to residential history was not suitable for inclusion in statistical analyses.

Finally, cases were excluded if they provided an insufficient volume of blood to conduct cultures with all antigens or insufficient supernatant was harvested to conduct all 13 cytokine ELISAs. This was essential to avoid biasing interpretation of cytokine profiles, particularly for analyses based on the combination of each participant's cytokine responses.

2.3.7 Determination of population age-infection profiles

Population estimates of *S. haematobium* infection intensity and prevalence were obtained from analysis of the 284 individuals who provided whole blood samples for the pre-treatment cross-sectional study. As is common in schistosome-endemic regions, infection intensity varied with age (Anderson and May 1992) as shown in Figure 2.5A. This distribution was convex in nature, with 11-12 year old children having the highest mean infection intensity when compared to other age groups within the population. 3 distinct age groups were identified from exploratory analysis reflecting age groups where infection levels are expected to increase with age (3-10 years), have reached a peak intensity (11-12 years) or

decline with age (13-86 years) according to this pattern (Figure 2.5B). The demographic and infection characteristics of these 3 groups are summarised in Table 1. These age groups are used throughout the study to relate cytokine responses to age and pre-treatment *S. haematobium* infection dynamics within Magaya community as a whole.

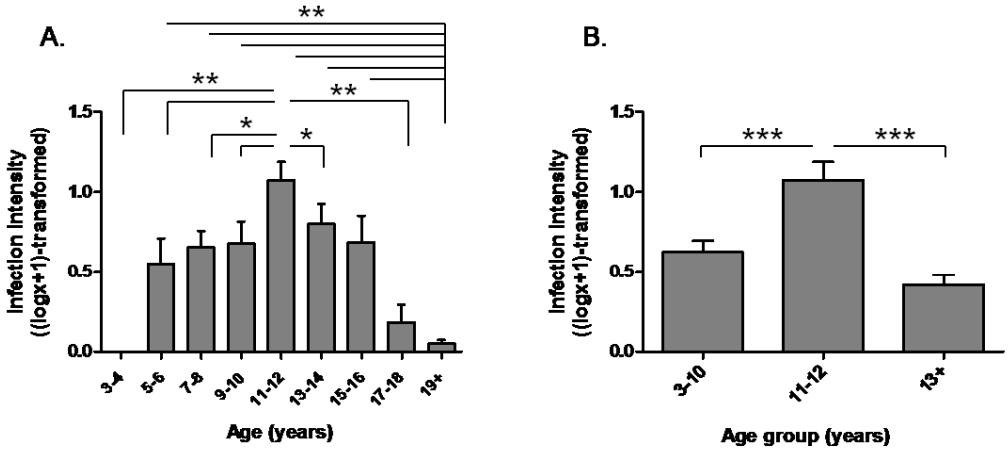


Figure 2.5. *S. haematobium* infection intensity by age in Magaya community (n = 284). A) Mean infection intensity is highest in 11-12 year olds and lowest in adults (aged 19+). B) The population-level distribution of infection is reflected in 3 age groups (3-10, 11-12 and 13+ years). Mean infection intensity (eggs/10ml urine) was compared between age groups (3-4, 5-6, 7-8, 9-10, 11-12, 13-14, 15-16, 17-18 and 19+ years (A) and 3-10, 11-12 and 13+ years (B)) by univariate analysis of variance with log10(x+1)-transformed infection intensity as the dependent variable and age group as the explanatory variable. *p<0.05, **p<0.01, ***p<0.001.

	Age group (years)		
	3 - 10	***11 - 12***	***13 +***
n	109	60	111
Mean age (S.E.M.)	7.4 (0.1)	11.5(0.07)	27.4 (1.7)
Number of males: females	52:57	31:29	45:66
Mean infection intensity (S.E.M)	24.0 (6.6)	68.7 (18.3)	12.6 (4.3)
Infection range (eggs/10ml urine)	0-481	0-692	0-403
Infection prevalence (%)	55.0	71.7	38.7

Table 2.2. Summary of demographic and *S. haematobium* infection characteristics of participants recruited from Magaya community (n = 284).

2.4 Cytokine responses of helminth-naïve participants experimentally exposed to *Trichuris suis* infection

The following study design and methods provide an overview of the clinical trial from which the samples analysed in chapter 7 were obtained (Bager *et al.* 2010a). Specific methods used for cytokine assays are provided in chapter 7.

2.4.1 Study design

This aspect of the study is part of a phase II randomised double-blind, placebo controlled clinical trial of *T. suis* ova (TSO) as an immunotherapy for allergic rhinitis (Registration number: R000001298, Trial ID: UMIN000001070). The trial was designed and implemented by Dr. Peter Bager and colleagues at the Serum Statens Institute, Copenhagen, Denmark, who provided plasma and PBMC supernatant samples collected during the study for cytokine analyses. The broader aim of the trial was to assess the efficacy of experimental *T. suis* infection on clinical symptoms of allergic rhinitis in a voluntary cohort of Danish individuals and these results have been recently published (Bager *et al.* 2010a).

Participants were recruited and randomly assigned to *T. suis* or placebo treatment groups and received 8 doses of 2500 TSO suspended in sulphate stabilised 0.015moles/L H_2SO_4 or the H_2SO_4 alone (placebo) in double-blinded preparations at 21 day intervals. Blood samples were collected at recruitment, during the peak grass pollen season and 21 days after the last treatment was administered. The grass pollen season was defined as the period from the first of 3 consecutive days where pollen counts reached (or exceeded) 10 pollen/m^3 (28^{th} May 2008) until the first of 3 consecutive days after the start of the season where pollen counts were below 10 pollen/m^3 (27^{th} July 2008). The design for the study is summarised in Figure 2.6. Although the date of sample collection during the grass pollen season varied between day 42 and 126 after initial treatment (Figure 2.6), 77% of the cohort received 3-5 doses prior to collection of grass pollen season serology (Bager *et al.* 2010b).

All participants were required to keep a patient diary throughout the study and report occurrence of asthma, diarrhoea, flatulence, pruritus ani, and any spontaneous reports of adverse events. Diarrhoeal episodes and gastric discomfort were assessed by daily

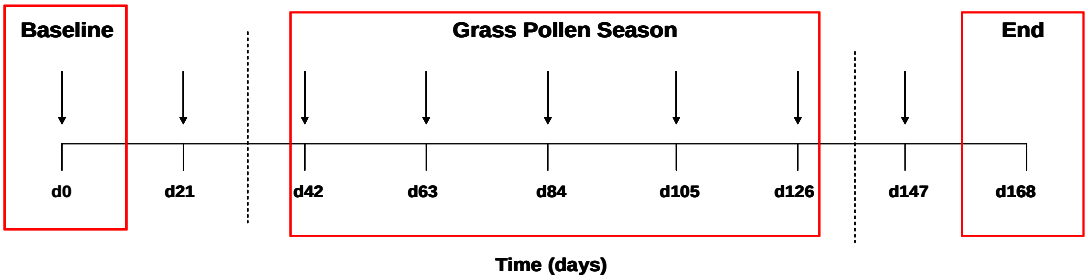


Figure 2.6. Study Design for phase II double-blind, placebo controlled clinical trial of *T. suis* ova as an immunotherapy for allergic rhinitis. Volunteers were randomly assigned to either TSO or placebo treatment groups and received 8 doses (indicated by arrows) of either 2500 TSO or placebo at 21 day intervals. Plasma and PBMC samples were collected at 3 timepoints (baseline, grass pollen season and end). The interval during which samples were collected for each timepoint is indicated by red boxes and the start and end of the grass pollen season are shown by dashed lines. d – number of days after ingestion of first treatment dose.

questionnaire. Data on self-reported GI symptoms (diarrhoea and discomfort) experienced between baseline and the peak grass pollen season and between the peak grass pollen season and the final timepoint were pooled into a single binary index (i.e. 0 – no gastrointestinal symptoms reported, 1 – GI symptom/s reported at any time after initial treatment). Daily, self-reported assessment of allergic rhinitis symptoms on a scale from 0 to 3 (0 – no symptoms, 1 – mild, 2 – moderate and 3 – severe symptoms), number of 'well days' and perceived improvement in symptoms compared to the grass pollen season of the previous year were used to assess the clinical efficacy of the treatment regime.

Preliminary results of the trial indicated that, although eosinophil counts and parasite-specific antibody titres were significantly elevated in the TSO-treated group relative to placebo controls, infection had no significant effect on clinical allergy (Bager *et al.* 2010a). The impetus for the current study was that sub-clinical changes in systemic, allergen and parasite-specific cytokine responses may yield insights into the immunobiology of experimental *T. suis* infection and identify a means of enhancing the clinical efficacy of the treatment regime.

2.4.2 Ethical considerations

Ethical permission to conduct the trial was obtained from the Danish Ethics Committee via independent review (number: H-KF-2006-4100). All volunteers provided written consent at recruitment and all aspects of the study accorded with the Declaration of Helsinki and Good Clinical Practice (GCP) (Bager *et al.* 2010a). Volunteers were free to drop-out of the study at any point and treatment was made available for GI and allergic symptoms if required. To ensure the safety of all participants, health assessment was monitored during visits and via telephone.

2.4.3 Study participants

Allergic rhinitis sufferes were recruited in Copenhagen, Denmark. All individuals suffered from seasonally exacerbated symptoms as a result of allergy to airborne pollen antigens (confirmed by skin prick test), levels of which peak in the Danish grass pollen season. 100 adult volunteers (age range: 19-63 years) provided a plasma sample and PBMCs were

isolated from 30 of these volunteers who were randomly selected prior to ingestion of the first treatment (baseline).

2.4.4 Methods

2.4.4.1 Serology

Venous whole blood samples were collected in anti-coagulant treated tubes for isolation of plasma by centrifugation and purification of PBMCs during the clinical trial conducted by Bager and colleagues (Bager *et al.* 2010a).

To complement these samples, raw data on eosinophil and lymphocyte counts, serum histamine levels, antigen-specific and total antibody titres over the course of the study were provided by Dr. Bager and colleagues. Eosinophil numbers were quantified by the Copenhagen G. P. Laboratory using differential cell counts slides. Total histamine (an indicator of basophil numbers) was assessed by lysis of whole blood cells with perchloric acid followed by spectroflurometric analysis using the glass microfiber protocol (RefLab ApS, Copenhagen, Denmark). Serum levels of grass pollen allergen-specific IgE, IgG and IgG4 and *T. suis* E/S-specific IgA, IgE, IgG and IgG4 were measured by ImmunoCAP (ISO 13485, Phadia AB, Uppsala, Sweden). These markers were chosen because peripheral blood eosinophilia and basophilia are characteristic of *T. suis* infection in pigs (Kringel and Roepstorff 2006) and IgA, IgE and IgG subclasses are prevalent in natural human trichiuriasis (Faulkner *et al.* 2002). IgE and IgG4 are associated with both allergic reactivity and helminth infection in humans (Yazdanbakhsh *et al.* 2002). This data has been independently analysed and published, confirming that antibody titres, cell counts, histamine levels and GI symptoms did not differ between the 2 treatment groups prior to treatment (Bager *et al.* 2010a). This was also the case when these parameters were compared by treatment group in the cohort included in cytokine analyses in the current study (Appendix 2).

2.4.4.2 Peripheral blood mononuclear cell (PBMC) re-stimulation cultures

PBMCs isolated from whole blood samples were cultured for 6 days with birch pollen allergen extract (t3, 15 µg/ml), timothy grass pollen allergen extract (g6, 15 µg/ml) or adult *T. suis* excretory-secretory products (E/S, 50 µg/ml) or left un-stimulated for control purposes. PBMCs were re-stimulated for a further 24 hours with PMA (25ng/ml) and ionomycin (1µg/ml) to enhance production of intracellular cytokines, after which cultures were centrifuged (5min, 500G) and the supernatants were harvested. All PBMC cultures were conducted by Bager and colleagues during the clinical trial(Bager *et al.* 2010a) and frozen culture supernatants were transported to the University of Edinburgh on dry ice for the cytokine analyses described in chapter 7.

2.4.4.3 Cytokine assays

IFNγ, TNFα, IL-4, IL-5, IL-10 and IL-13 were measured in plasma and PBMC culture supernatants and total (heat-activated) TGFβ was measured in the PBMC culture supernatants. This panel of cytokines was chosen to characterise responses associated with exacerbated allergy and *T. suis* infection. The Th2-type cytokines IL-4 and IL-13, Th1-type cytokine IFNγ and innate inflammatory cytokine TNFα are known to be up-regulated in natural human *T. trichiura* infection (Faulkner *et al.* 2002). IL-4 and IL-13 are also mediators of the allergen-induced late-nasal response in allergic rhinitis (Ghaffar *et al.* 1997). IL-5 was included in the panel as it is associated both with resistance to re-infection with *T. trichiura* (Jackson *et al.* 2004a) and exacerbation of Th2-mediated allergic pathology (Fallon and Mangan 2007). The immunomodulatory cytokines IL-10 and TGFβ have also been shown to play an important role in dampening immune activation in natural human helminth infections (Doetze *et al.* 2000; Figueiredo *et al.* 2010).

IFNγ, TNFα, IL-4, IL-5, IL-10 and IL-13 were quantified via cytometric bead array (CBA). CBA uses a combination of cytokine-specific antibody conjugated capture beads and cytokine-specific phycoerythrin (PE)-conjugated detection antibody to quantify cytokines in biological samples. This method differs from ELISA because the unique size and emission wavelength of 2-fluorescence markers (Red and Near Infra-red) of each cytokine-specific capture bead population allows multiple cytokines to be assayed simultaneously in a single sample and quantified independently using flow cytometry. The sensitivity of CBA is equivalent to that of a sandwich ELISA (BDBiosciences 2009), but the requirement for lower volumes of sample meant that this method was ideal for maximising the number of

cytokines that could be assayed in the sample aliquots obtained from the clinical trial. For full details of the CBA protocol used, see chapter 7.

TGFβ was measured in the same t3, g6, E/S and un-stimulated PBMC culture supernatant aliquots as used for CBA cytokine analysis. A bioassay was used to quantify active TGFβ rather than the inactive latent form and details of the method used are provided in chapter 7.

2.5 Statistical methods

In the current study I have used multivariate statistical methods to characterise cytokine profiles and immune phenotype rather than focusing on individual cytokines in isolation. Detailed methods are described in relevant chapters; however an overview of my statistical approach is described here.

2.5.1 Parasitology data

It is well established from epidemiological surveys of schistosome infection that egg counts are highly heterogeneous within human populations and tend to be aggregated in a minority of individuals (reviewed by (Mutapi and Roddam 2002)). Thus arithmetic mean schistosome infection intensities were log10(x+1) transformed to meet the assumptions of parametric tests where possible (Sokal and Rohlf 1995c). For all parametric analyses of schistosome infection intensity the distribution of residuals from statistical models and group variances were compared for both un-transformed and transformed data to check that the assumption of normality and equality of variance were met and that the data transformation was appropriate for the analysis.

2.5.2 Cytokine data

Assaying multiple cytokines presents an analytical challenge for a number of reasons: 1) antigen-specific cytokine responses measured in the same individual are not independent since measured concentrations are influenced by spontaneously secreted levels of non-specific cytokines, which vary between individuals, 2) cytokine responses to the same

antigen within a single sample are not independent of one another as they both mediate and are influenced by cellular interactions, 3) concentrations are not directly comparable between different types of cytokines due to their distinct bioactivities (determined by cellular expression of cytokine receptors, presence of cytokine receptor antagonists and immunoregulatory molecules), 4) different cytokine responses vary greatly in their secretion patterns with some found to be produced at almost ubiquitously high concentrations (e.g. IL-6 and IL-8) and others consistently produced in low or negligible amounts (e.g. IL-4 and IL-17A) and 5) multiple comparisons between cytokine responses must be accounted for in analyses to avoid type I error (Holm 1979; Rice 1989).

To address these issues I have used a number of procedures throughout the study. Firstly, I have subtracted levels of non-specific cytokines (measured in parallel un-stimulated cultures) from those measured in antigen-specific cultures to ensure that responses to different antigens can be compared as independent variables (Mutapi and Roddam 2002). This approach has been used for analysis of antigen-specific cytokine in a number of previous human field studies (Remoué *et al.* 2001; Mutapi *et al.* 2007b; Wilson *et al.* 2008). So that non-specific cytokine dynamics are not ignored I have also analysed responses in un-stimulated cultures where relevant.

To facilitate combined analysis of multiple cytokine responses in the context of their inter-dependency, aggregated secretion patterns within populations and the quantitative differences between types I have used data reduction (factor analysis) and ordination methods (non-metric multi-dimensional scaling (NMS)) adapted for use in heterogeneous data sets with variables measured on a range of different scales (Sokal and Rohlf 1995a; McCune and Grace 2002). These methods (described below) provide an ideal means of identifying patterns of cytokine responses because they group cytokine variables according to their co-variance with each other(factor analysis) (Sokal and Rohlf 1995a) and relative dissimilarities between participants (NMS) (McCune and Grace 2002) rather than by their absolute quantities. Reducing cytokines into a smaller number of variables via factor analysis and a combined cytokine NMS have the added benefit of reducing the number of comparisons made and thus reduce the risk of false positive results. These methods are used routinely in psychometric (Kruskal 1964; Harris 1967) and population ecology (McCune and Grace 2002) analyses respectively.

To account for multiple comparisons made in statistical tests I have used the sequential Bonferroni adjustment of the significance level ($p<0.05$) for each set of comparisons made (Holm 1979; Rice 1989). The sequential rather than standard adjustment was chosen because it imposes less risk of false negative results but retains statistical power to reject false positive results (Rice 1989). This method involves ranking the p-values of all comparisons made (smallest to largest) and adjusting the chosen significance level (0.05) for each comparison by dividing by its rank (Rice 1989). The Bonferroni adjustment is conducted for all tests where more than 5 comparisons are made using a statistical test and adjustments are 'table-wide' (i.e. where test statistics are reported in the same table, the Bonferroni adjustment is made across all tabulated p-values). In all cases raw p-values for tests are reported and discussed as an indicator of an observed trend and those significant after adjustment are highlighted.

To meet the assumption of linearity for parametric tests I have used statistical transformations of cytokine data where possible. In most cases the square-root(x+1) transformation was found to most effectively render aggregated cytokine concentrations suitable for parametric analysis (Sokal and Rohlf 1995c; Mutapi and Roddam 2002; Osborne 2002). Addition of a constant (i.e. 1) prior to square-root transformation also allowed individuals negative for one or more cytokine to be included in analyses (Osborne 2002). For all statistical models the distribution of residuals obtained from analysis of both un-transformed and transformed data was checked for normality and the variance of groups was compared to check that the assumption of equality of variance was met. Where transformed data did not meet the assumptions of parametric tests I have used appropriate non-parametric alternatives to analyse un-transformed data.

2.5.3 Factor analysis

I have used factor analysis to reduce multiple cytokines responses into a smaller number of variables reflecting the groups of cytokine responses that show similar secretion patterns between individuals (chapters 3, 4, 5 and 6). The variables extracted by this analysis, called principal components (PCs), can be considered analogous to distinct phenotypic immune responses or 'cytokine profiles' within the parasite-specific cytokine milieu. These phenotypes were interpreted for each PC according to the innate inflammatory, Th1, Th2, Th17, pro-inflammatory and regulatory cytokine groupings identified via the literature

review described in chapter 2.3.5.5. PC regression factor scores for each participant were subsequently analysed in statistical models.

Square-root(x+1) transformed cytokine data was used for factor analysis throughout the study as exploratory analysis indicated that transformation improved the fit of the data to subsequent statistical models (assessed by comparing the R^2 values of each model) without altering the groupings of the cytokine variables relative to un-transformed data. Monotonic transformation of raw data in this way is also recommended prior to factor analysis to reduce the weight of outliers, which might bias interpretation of the extracted PCs (Rummel 1970).

The factor analysis procedure is described in detail elsewhere (Sokal and Rohlf 1995a). Briefly, the cytokine responses of the study cohort are grouped according to their linear correlations into a smaller number of un-correlated variables (PCs). Each PC accounts for a proportion of variation in the cytokine responses between the cohort as a whole with PC1 accounting for the most variation and subsequent PCs accounting for sequentially lower amounts of the remaining variation. Only PCs which accounted for a greater than average proportion of variance in the original cytokine data (eigenvalue >1) were included in subsequent analyses (Kaiser 1960). The cytokines constituting each PC can be identified by their factor loading, which is the correlation coefficient between that cytokine response and the new variable (PC). PCs reflect the patterns of cytokine responses with which they are positively correlated and the opposite pattern to cytokines with which they are negatively correlated. Where 2 cytokine responses load onto the same PC with different directions of correlation, their secretion patterns are considered to be reciprocal. Only PCs that correlated with at least 2 of the original cytokine variables with a factor loading of greater than 0.5 or less than -0.5 were included in subsequent analyses. These cut-off values were chosen because they equate to at least 25% of the variance in a factor positively/negatively correlating with the extracted PC. The result is that each participant is assigned a regression factor score (the sum of a participant's standardised score for each cytokine multiplied by the factor loading of the PC) for each PC (cytokine profile). All factor analyses were conducted in SPSS software.

I have used 2 main types of factor analysis to group cytokine variables according to immune phenotype. Firstly, where the purpose was to subsequently compare the cytokine profiles elicited by different antigens in the same individuals (chapters 3.3.2 and 5.4.1), each

participant was included separately for each antigen stimulation (CAP, WWH and SEA-specific responses). Thus part of the variation explained by the extracted PCs was due to differences between antigens, allowing their comparison. Secondly, where the purpose was to relate cytokine profiles to variation between different groups of individuals (chapter 4 and 6), each participant was included once but cytokine responses to different antigens were included as separate variables for that individual. Thus the extracted PCs would allow antigen-specific responses of each individual to be collectively related to other variables. Only cytokine responses to crude parasite antigen preparations (*S. haematobium* CAP, WWH and SEA) were considered suitable for inclusion in the same factor analysis because each contains a physiologically-relevant mixture of antigens (i.e. derived from whole parasites), some of which will be expressed by more than one of the 3 life-cycle stages(Curwen *et al.* 2004). *S. haematobium* GST-specific cytokines were analysed separately because they reflect responses to a single recombinant antigen and because stimulation was conducted using the antigen at concentrations in excess of that expected to be present in a natural infection.

2.5.4 Non-metric multi-dimensional scaling

NMS is an ordination technique that allows qualitative patterns in participants' cytokine responses to be identified according their similarity/dissimilarity to other participants and their own cytokine profiles at different timepoints. NMS also produces a comprehensive visual representation of these variations via ordination plots allowing patterns of cytokine responses between individuals to be qualitatively characterised by how close together (similar) or far apart (dissimilar) they are. Unlike factor analysis, which assumes that variables are linearly related, NMS does not assume linear relationships between participants or cytokine responses (McCune and Grace 2002), making it more suitable for comparison of different timepoints. In particular I have used NMS to investigate how cytokine responses change with time: pre and 6 weeks post-treatment for the *S. haematobium* study (chapter 5) and baseline, grass pollen season and end timepoints for the *T. suis* study (chapter 7).

Prior to NMS cytokine concentrations were square-root(x+1)-transformed to reduce the weight of outliers on the ordination solutions without affecting relative ranks of each participant (Rummel 1970), as has been effectively used previously (Moore-Kucera and Dick 2008; Walker 2008; Turcotte *et al.* 2009).

Full details of the NMS procedure are described elsewhere (McCune and Grace 2002). Firstly, the Sorensen distance (a measure of how similar/dissimilar one participant is from another) was calculated for each participant according to the rank of each of their cytokine responses relative to those of other participants (McCune and Grace 2002). Sorensen distance was chosen for NMS because it is sensitive to patterns in heterogeneous data sets and assigns less weight to outlier values than alternative distance measures (McCune and Grace 2002). Sorensen values were then used to position each participant relative to all other participants at all timepoints on 2 dimensional axes (i.e. each participant is plotted at all timepoints on a single plot). The axes along which participants' cytokine profiles at different timepoints are plotted reflect cytokine responses that account for the greatest variation within the data set as a whole. Axes values are arbitrary since NMS ordination plots characterise patterns of cytokine responses spatially rather than quantitatively. All NMS analyses were conducted in PC-ORD software.

Since NMS is an iterative process, the final ordination plot achieved can vary (particularly if patterns of cytokine responses are weak) and depends upon the starting configuration of the analysis. Thus 2-dimensional NMS ordination was initiated with random starting configurations and repeated a minimum of 5 times with different starting configurations to ensure that the final solution reliably reproduced patterns of participant cytokine responses. Final ordination plots were obtained after 100 runs with the cytokine data and a maximum of 500 iterations with a stability criterion of 0.000001, an adaptation of the default software settings selected to increase the accuracy of the final solution (recommended by (McCune and Grace 2002)) and verified by comparison of final stress values using a range of different settings. Ordination solutions were considered to reliably represent patterns of cytokine responses if a final stress value (a measure of the difference between the rank of a participant's original cytokine responses versus that calculated during NMS ordination (McCune and Grace 2002)) of less than 20 was achieved. Monte-Carlo randomisation tests were also conducted to ensure that spatial patterns identified in the cytokine data were stronger than those that would result from the same data if it were randomly assorted (McCune and Grace 2002). 20 runs of randomly assorted data were used to select initial parameters and 50 runs were used for final analyses to give a 'slow and thorough' assessment of the NMS parameters (McCune and Grace 2002).

To identify how ordination patterns related to variation in the original cytokine data the proportion of variance represented by each axis (i.e. the coefficient of determination (r^2) between the original cytokine matrices and the ordination space generated by NMS) was calculated to identify how much variation between participants was accounted for by each axis. Clusters of participant cytokine responses relative to these axes were identified visually. The Pearson's correlations between the individual cytokine responses and each axis were then calculated to identify the cytokines responsible for the observed variation between participants. In the latter case only cytokines with an $r^2>0.5$ were considered to contribute to contribute to the axes.

2.5.5 Multiple-response permutation procedure (MRPP)

MRPP is a non-parametric alternative to multivariate ANOVA and is more suitable for multivariate analysis of heterogeneous data, such as Sorensen distance values used for NMS (McCune and Grace 2002). MRPP was conducted using PC-ORD software.

MRPP is a step-wise process, described in detail in (McCune and Grace 2002). Firstly, the sum of Sorensen distances between participant cytokine responses is calculated for each group (e.g. treatment, timepoint or antigen stimulation) (see 2.5.4) to give the weighted mean within-group distance (δ). Mean within-group differences reflect the total variation between participant cytokine responses in each group. A test statistic (T) is then calculated by dividing the difference between the observed δ and the δ expected under the null hypothesis (no difference) divided by the expected standard deviation. Thus larger values of T indicate a greater difference between the groups being compared and the significance of this difference can be determined by its associated p-value. Finally, homogeneity in cytokine profiles within groups is quantified via the chance corrected within-group agreement statistic (A = 1-(observed δ/expected δ)), an estimate of 'effect-size' for the between-group difference identified by MRPP. When participants within a group exhibit the same cytokine profile and groups differ considerably the value of A is closer to 1 (if all participants cytokine profiles were identical after treatment, A = 1 (McCune and Grace 2002)). This can be visualised on an NMS ordination plot as participants in different groups forming distinct clusters with little overlap. Alternatively if cytokine profiles are highly heterogeneous and show little structure according to treatment, A is closer to 0 (if heterogeneity within the pre and post-treatment groups is equal to that expected by chance, A = 0 (McCune and Grace 2002)). On an NMS

ordination plot this visualised as indistinct clusters and overlap between participants in the different groups. Given the size of our study cohort, an A <0.1 was considered as a small and A> 0.3 was considered a large effect size (McCune and Grace 2002).

Chapter 3

Life-cycle stage-specific cytokine responses to *Schistosoma haematobium* in a naturally exposed human population

3.1 Introduction

Given their distinct exposure patterns, intra-host behaviour, proteomes (Curwen *et al.* 2004) and gene expression patterns (Jolly *et al.* 2007; Fitzpatrick *et al.* 2009) it seems likely that cercariae, adult worm and egg-stage schistosomes also differ in their immunobiology. Crude antigen preparations of these 3 life-cycle stages are known to elicit distinct antibody responses (Viana *et al.* 1995) and a number of studies suggest that there are also differences in the cytokine responses that they elicit (see chapter 1.5.1). However, investigations conducted to-date provide an incomplete picture of the human cytokine responses to the different life-cycle stages of *S. haematobium* due to 3 main limitations:1) a bias towards Th1 and Th2-associated cytokine responses and an absence of data on schistosome-specific Th17-associated cytokines, despite evidence that this lineage may influence the development of human helminth-associated disease (Babu *et al.* 2009; Milner *et al.* 2010), 2) a predominant focus on schistosome eggs and adult worms, but few recent studies of cercariae-specific cytokine responses and 3) analysis of individual parasite-specific cytokine responses without accounting for the multivariate interactions between them. The latter is particularly limiting when a small panel of cytokines are assayed and interpreted in terms of specific CD4+ T cell phenotypes since human peripheral immune responses are less clearly defined than those of murine models (from which many immunological paradigms are translated) and even cytokines associated with the same phenotype can dissociate in response to schistosome antigens (Grogan *et al.* 1996a; Scott *et al.* 2000).

The aim of this chapter is to provide a systematic analysis of schistosome-specific cytokine responses in whole blood samples collected from endemically-exposed humans. To address the limitations of previous cytokine studies I have assayed responses to cercariae, adult worm and egg antigens and quantified cytokines associated with both innate and acquired

cellular immune responses, including Th1, Th2 and for the first time in human schistosomiasis haematobium, Th17-type cytokines. Thus it was possible to test the hypothesis that cercariae, adult worm and egg stage parasites elicit not only distinct amounts of individual cytokines, but also elicit distinct profiles of cytokines reflecting differences in the way that they polarise cellular immune responses. The results of this chapter provide an indicator of how exposure to different *S. haematobium* life cycle stages may influence the immune environment of their host.

3.2 Hypotheses

- Schistosome antigens elicit cytokines associated with Th17-type responses in addition to innate inflammatory, Th1 and Th2-type cytokines

- *S. haematobium* cercariae, adult worm and egg antigens differ both in the levels of individual whole blood cytokines and the cytokine profiles that they elicit *in vitro*

3.3 Materials and Methods

3.3.1 Study participants

Of the initial cross-sectional cohort recruited from Magaya community only those who met the following inclusion criteria are included in the current chapter: 1) provided adequate samples for parasitology, to enable reliable detection of *S. haematobium* infections and co-infections, 2) were negative for all co-infections assayed (*S. mansoni*, soil-transmitted helminths (STH), malaria and human immunodeficiency virus (HIV)), which are known to influence immune responses to schistosome antigens (chapter 1.4.5 and 2.3.6) and 3) had never received treatment for schistosomiasis, which is known to alter naturally acquired immune responses to schistosome antigens (chapter 1.7.3). Since the study aimed to directly compare cytokine responses to cercariae, adult worm and egg antigens, participants were also excluded if their blood sample had not been cultured with all three antigen preparations.

56 individuals (both infected and un-infected) met the above selection criteria and their demographic and infection characteristics are summarised in Table 3.1.

	Males (n = 26)		***Females (n = 30)***		***Total (n = 56)***	
	Mean (S.EM.)	***Range***	***Mean (S.E.M.)***	***Range***	***Mean (S.E.M.)***	***Range***
Age (years)	9.35 (0.266)	8 – 12	22.41 (3.93)	8 - 84	16.24 (2.24)	8 - 84
Infection intensity	53.82 (28.36)	0 – 693	10.01 (5.639)	0 - 158	30.35 (13.69)	0 - 693
Prevalence (%)	53.85		36.67		44.64	

Table 3.1. Characteristics of the study cohort for comparison of life-cycle stage-specific cytokine responses to *S. haematobium*. Infection intensity was quantified as mean *S. haematobium* egg counts/10ml urine. S.E.M. – Standard error of the mean.

3.3.2 Immunological assays

All individuals provided a whole blood sample which was stimulated for 48 hours at 37°C with 10µg/ml crude preparations of *S. haematobium* cercariae (CAP), adult worms (WWH) and eggs (SEA) in parallel cultures. Cultures stimulated with 2µg/ml maltose binding protein (MBP) indicated the ability of cultured whole blood cells to respond to non-schistosome antigens. Whole blood cultured with media alone (un-stimulated) acted as negative controls for levels of spontaneous cytokine. Cytokines associated with innate inflammatory (TNFα, IL-6 and IL-8), Th1 (IFNγ, IL-2 and IL-12p70), Th2 (IL-4, IL-5, IL-10 and IL-13) and Th17 (IL-17A, IL-21 and IL-23p19)-type responses were assayed in culture supernatants by enzyme-linked immunosorbent assay (ELISA) and a mean concentration (ng/ml) was obtained from duplicate ELISA wells. The association of individual cytokine responses with cellular immune phenotypes was determined by a review of current literature on human cytokine responses (summarised in chapter 2.3.5.5). The magnitude of cytokine responses to MBP and in un-stimulated cultures are shown in Figure 3.1, which demonstrates that all selected participants produced one or more of the assayed cytokines in response to MBP stimulation and that pro-inflammatory cytokine responses to MBP were above background levels (un-stimulated cultures).

3.3.3 Statistical Analyses

Antigen-specific cytokine responses were obtained by subtracting cytokine concentrations present in un-stimulated cultures from those present in antigen-stimulated cultures so that CAP, WWH and SEA-specific cytokine responses could be analysed as independent variables. Antigen-specific cytokine responses were compared between exactly the same individuals to control for potential variation according to age, sex, genetic factors, infection intensity, residential or exposure history. Comparisons were made using data on all 13 cytokines to all 3 antigens.

Exploratory analysis indicated that the distribution of cytokine concentrations was highly skewed and the variance in the cytokine responses to the 3 antigen preparations was non-homogenous even after statistical transformations. Therefore, to test the hypothesis that individual

cytokine responses differ according to *S. haematobium* life-cycle stage, the non-parametric paired Wilcoxon signed-rank test was used to compare ranks of un-transformed supernatant

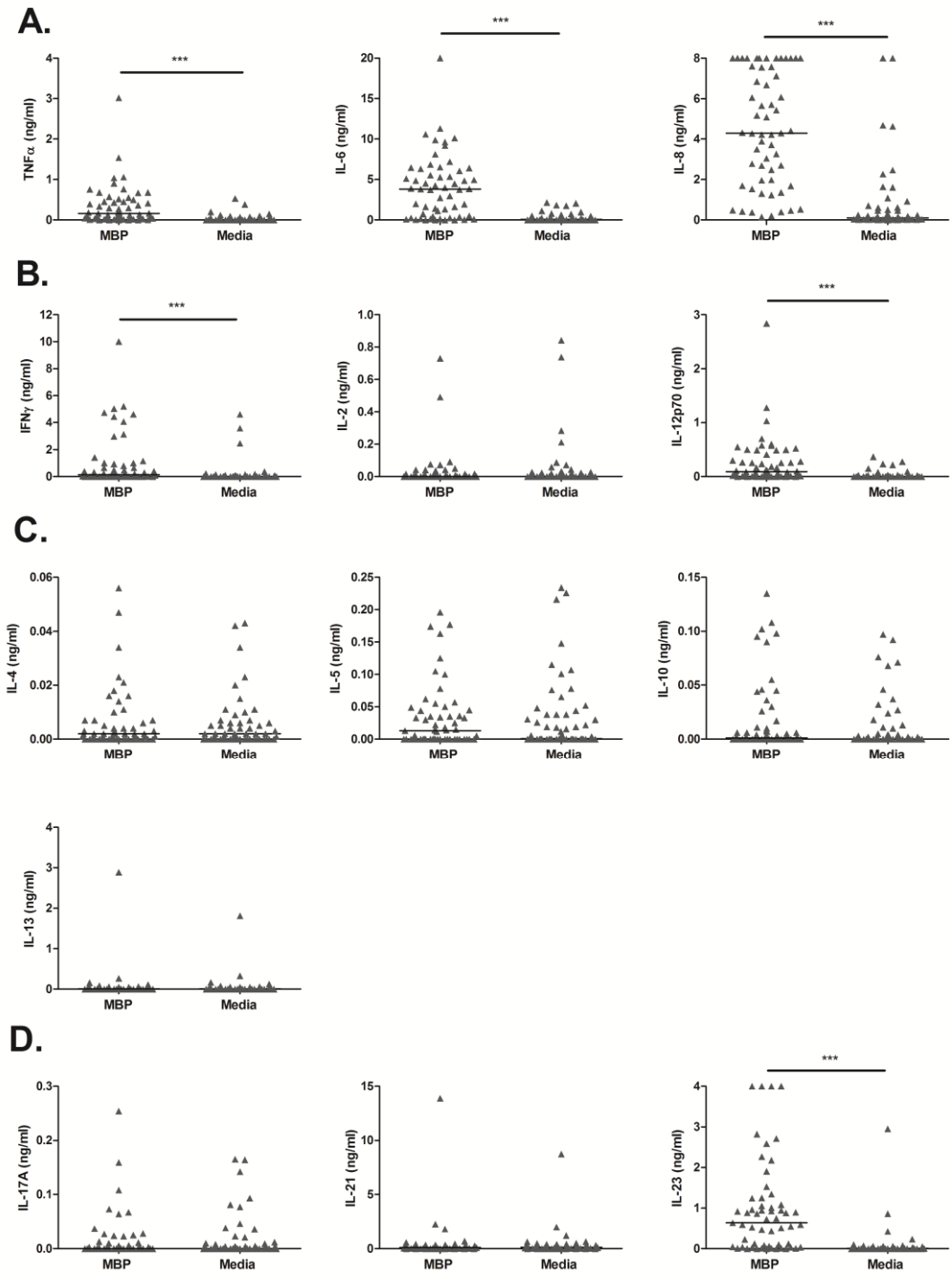


Figure 3.1 Levels of cytokines present in whole blood cultures stimulated with Maltose-binding protein (MBP) and those cultured with media alone (un-stimulated). Raw concentrations of innate inflammatory-(A), Th1-(B), Th2-(C) and Th17(D)-type cytokines assayed in whole blood supernatants (n = 56) and median values are indicated. Results of Wilcoxon comparisons are shown. ***p<0.001.

cytokine levels (Wilcoxon 1945).

To characterise the cytokine profile elicited by the different parasite life-cycle stages, all 13 square-root(x+1)-transformed CAP, WWH and SEA-specific cytokines were reduced into groups by factor analysis (Sokal and Rohlf 1995c). Each participant's cytokine response to all 3 antigens was included in the same analysis so that variation within each of the extracted principal components (PCs) included variation in the cytokine profiles due to antigen stimulation. The factor analysis procedure is described in detail in chapter 2. 5.3. Only PCs accounting for a greater than average proportion of variation in cytokine responses (Kaiser 1960) and loaded with 2 or more of the original cytokine variables with factor loadings greater than 0.5 or less than -0.5 were included in subsequent analyses.

To investigate whether cytokine profiles differed between cercariae, adult worm and egg-stage antigens analysis of variance (ANOVA) of the PC regression factor scores for each cytokine profile was used. Regression factor scores for all PCs were entered as dependent variables and antigen (CAP, WWH and SEA) was included as the explanatory variable. Exploratory analysis indicated that the residuals of all PC regression factor scores from ANOVA models were normally distributed and the variance in CAP, WWH and SEA-specific scores were equal and thus the data met the assumptions of parametric testing. Post-hoc pair-wise comparisons between antigens were conducted using Fisher's least significant difference test (Sokal and Rohlf 1995b).

For all statistical tests cytokine profiles were considered to differ significantly according to antigen stimulation if $p<0.05$.To provide an indicator of the effect of multiple comparisons on the significance of the difference between antigen stimulations the significance level was adjusted for all tests using the sequential Bonferroni method (Holm 1979; Rice 1989) (see 2.5.2 for full details). Raw p-values are reported and their significance after Bonferroni correction indicated where relevant.

3.4 Results

3.4.1 Individual cytokine responses to *S. haematobium* cercariae, adult worm and egg antigens

To test the hypothesis that *S. haematobium* life-cycle stage antigens elicit different levels of individual cytokine responses within the cohort concentrations of each of the 13 cytokines assayed were compared by antigen stimulation (CAP, WWH and SEA). The highest mean concentrations of different cytokines are clearly segregated according to both antigen stimulation and the cellular immune phenotype with which the cytokines are associated. WWH did not elicit higher mean concentrations of any cytokine than either CAP or SEA within the cohort. A summary of the relative mean concentrations of each cytokine elicited by each antigen is given in Table 3.2.

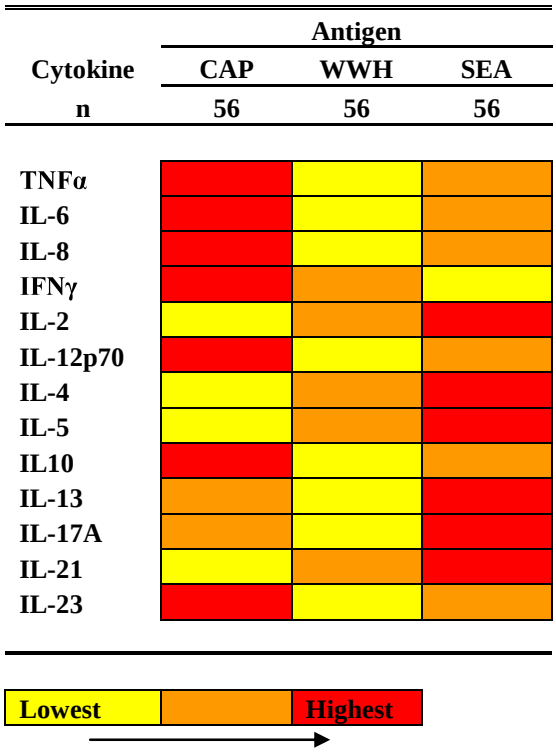


Table 3.2. Summary of differences between mean whole blood cytokine concentrations elicited by *S. haematobium* cercariae (CAP), adult worm (WWH) and egg (SEA)-specific antigen preparations. Colours indicate whether mean cytokine concentrations were highest (red), intermediate (orange) or lowest (yellow) relative to parallel cultures conducted in the same individuals using the other 2 crude homogenate antigens.

CAP-specific IFNγ, TNFα, IL-6, IL-12p70 and IL-23 responses were significantly greater than those produced in response to either SEA or WWH. IL-8 and IL-10 were significantly greater in CAP-stimulated cultures than in WWH-stimulated cultures, but the difference in IL-10 was not significant after Bonferroni correction. SEA-stimulated cultures had higher IL-6 (not significant after Bonferroni correction) and IL-8 than WWH-stimulated cultures. IL-2, IL-4, IL-17A and IL-21 were significantly higher in SEA relative to CAP-stimulated cultures although these differences were not significant after Bonferroni correction for multiple comparisons. Of the cytokines assayed, IL-17A was detectable in few participants and median levels in response to CAP, WWH and SEA were ≤0.01ng/ml above concentrations present in un-stimulated cultures. Collectively these patterns indicate that cercarial antigens elicit higher levels of pro-inflammatory effector cytokines than adult worms or eggs. In contrast, eggs elicit higher levels of a distinct sub-set of cytokines than CAP, suggesting that the 2 life-cycle stages polarise the immune-response differently.

Un-transformed life cycle stage-specific cytokine responses of the cohort are plotted for each individual cytokine and plots are grouped as innate inflammatory (Figure 3.2), Th1-type (Figure 3.3), Th2-type (Figure 3.4) or Th17-type (Figure 3.5) cytokines. Z values and the associated significance levels obtained from paired Wilcoxon signed-rank test comparisons of individual cytokine responses to each of the stage-specific antigen preparations are given in Table 3.3.

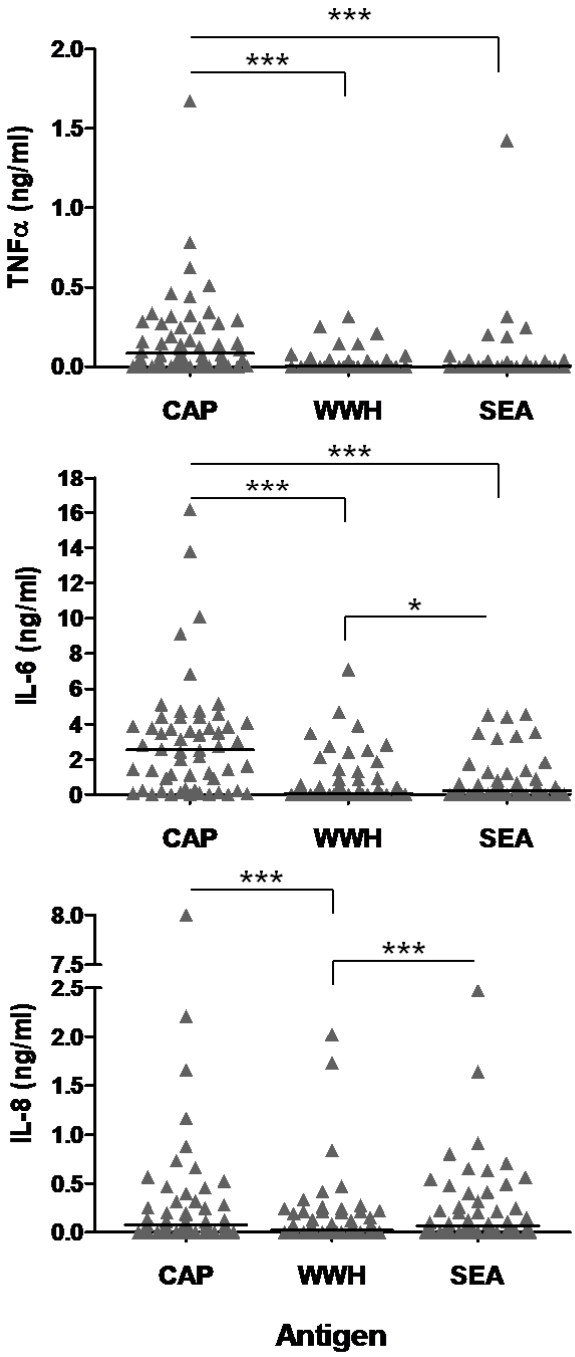


Figure 3.2. Innate inflammatory-type cytokine responses differ between *S. haematobium* cercariae, adult worm and egg stage antigens. Graphs show un-transformed mean concentrations (ng/ml) produced by whole blood cells in response to stimulation with cercariae (CAP), adult worm (WWH) or egg (SEA) antigens. Median values are indicated by black lines. Ranked cytokine concentrations were compared between antigens via paired Wilcoxon signed rank test. *p<0.05, ***p<0.001

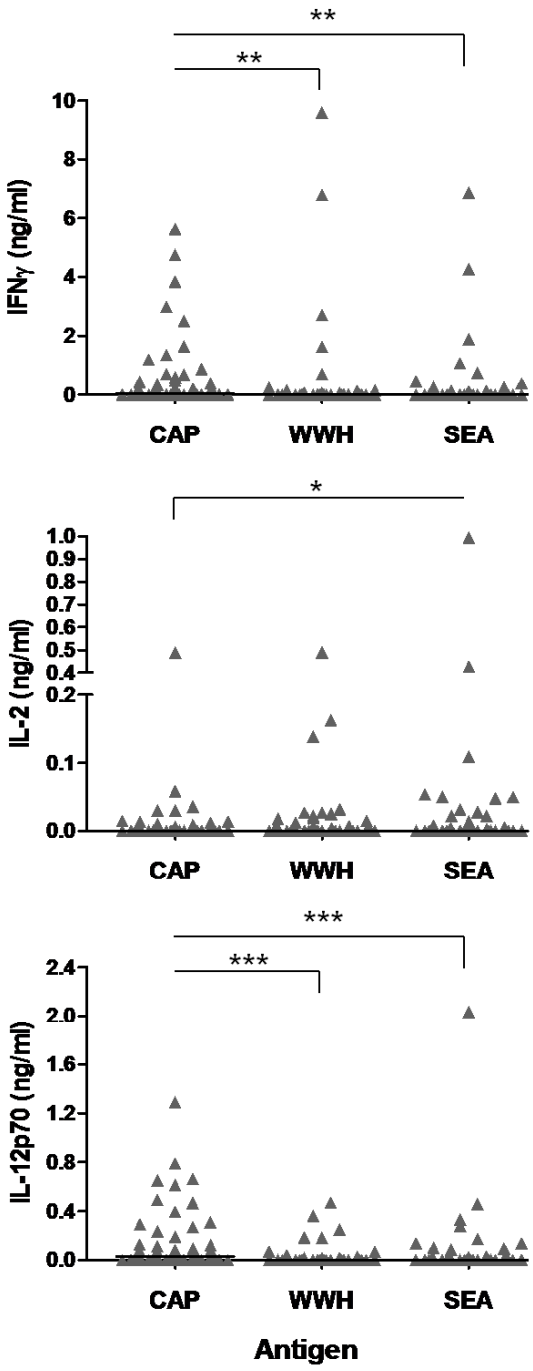


Figure 3.3. Th1-type cytokine responses differ between *S. haematobium* cercariae, adult worm and egg stage antigens. Graphs show un-transformed mean concentrations (ng/ml) produced by whole blood cells in response to stimulation with cercariae (CAP), adult worm (WWH) or egg (SEA) antigens. Median values are indicated by black lines. Ranked cytokine concentrations were compared between antigens via paired Wilcoxon signed rank test. *p<0.05, **p<0.01, ***p<0.001

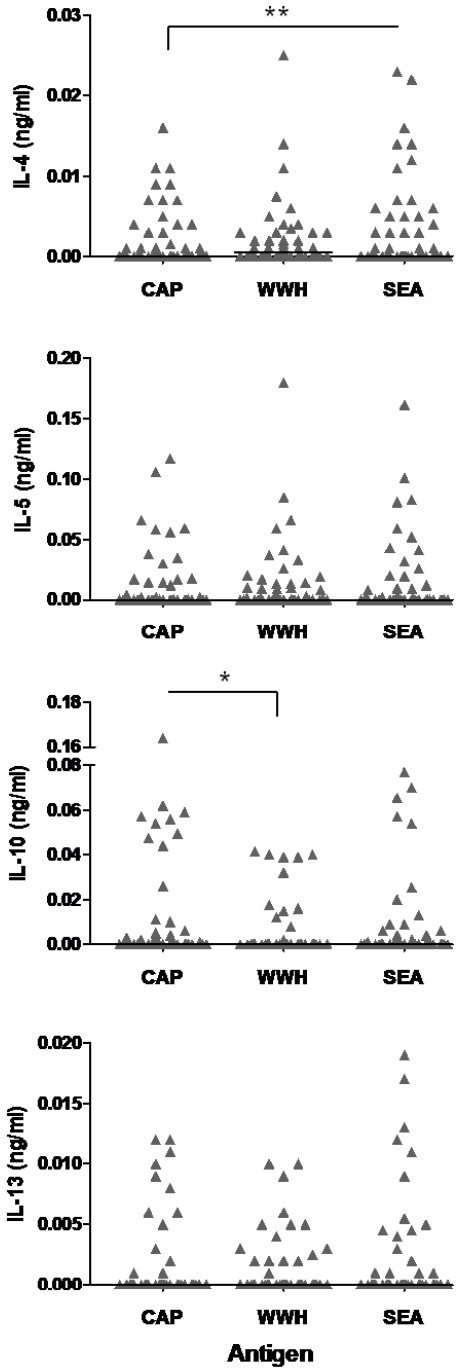


Figure 3.4. Th2-type cytokine responses differ between *S. haematobium* cercariae, adult worm and egg stage antigens. Graphs show un-transformed mean concentrations (ng/ml) produced by whole blood cells in response to stimulation with cercariae (CAP), adult worm (WWH) or egg (SEA) antigens. Median values are indicated by black lines. Ranked cytokine concentrations were compared between antigens via paired Wilcoxon signed rank test.*$p<0.05$, **$p<0.01$

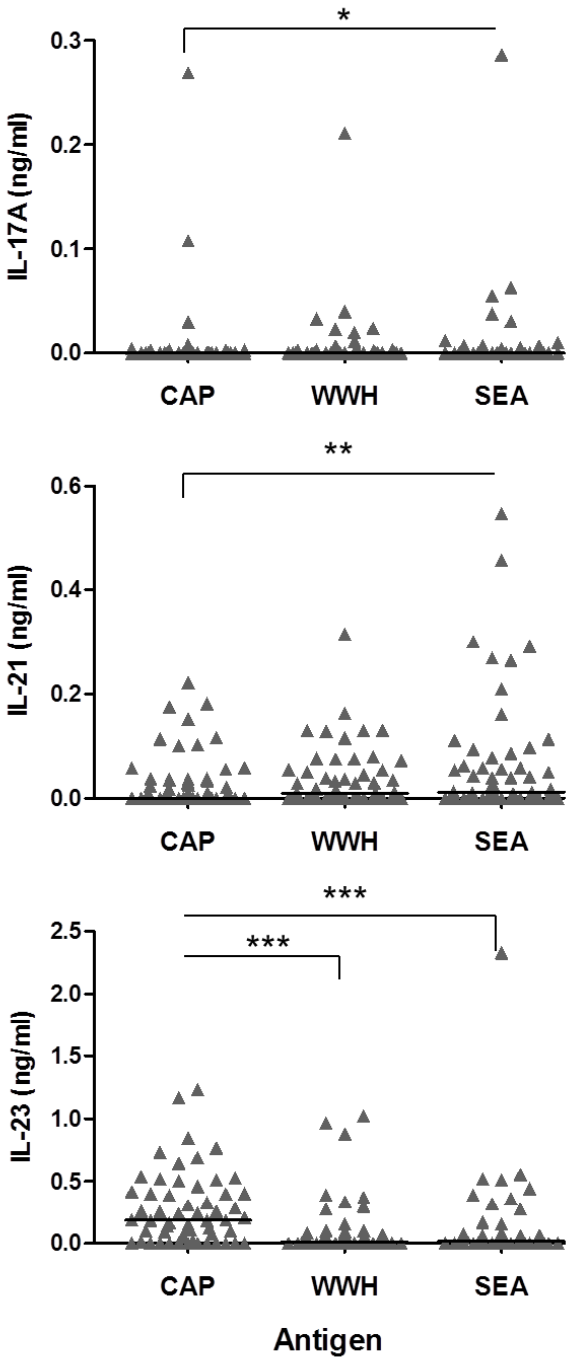


Figure 3.5. Th17-type cytokine responses differ between *S. haematobium* cercariae, adult worm and egg stage antigens. Graphs show un-transformed mean concentrations (ng/ml) produced by whole blood cells in response to stimulation with cercariae (CAP), adult worm (WWH) or egg (SEA) antigens. Median values are indicated by black lines. Ranked cytokine concentrations were compared between antigens via paired Wilcoxon signed rank test. *p<0.05, **p<0.01, ***p<0.001

		Innate Inflammatory			*Th1*			*Th2*				*Th17*		
		TNFα	***IL-6***	***IL-8***	***IFNγ***	***IL-2***	***IL-12p70***	***IL-4***	***IL-5***	***IL-10***	***IL-13***	***IL-17A***	***IL-21***	***IL-23***
CAP vs. WWH	***Z***	-5.037	-5.998	-4.564	-2.738	-0.205	-5.398	-0.241	-0.380	-2.582	-0.436	-0.711	-1.086	-4.794
	Sig.	**<0.001**	**<0.001**	**<0.001**	**0.006**	0.837	**<0.001**	0.810	0.704	**0.010**	0.663	0.477	0.278	**<0.001**
CAP vs. SEA	***Z***	-4.985	-5.678	-1.288	-3.098	-2.087	-4.429	-2.615	-0.552	-0.928	-0.956	-2.458	-2.793	-4.595
	Sig.	**<0.001**	**<0.001**	0.198	**0.002**	**0.037**	**<0.001**	**0.009**	0.581	0.353	0.339	**0.014**	**0.005**	**<0.001**
WWH vs. SEA	***Z***	-0.638	-2.422	-3.551	-1.546	-1.279	-1.680	-0.380	-0.067	-1.154	-1.627	-1.015	-1.297	-1.179
	Sig.	0.524	**0.015**	**<0.001**	0.122	0.201	0.093	0.704	0.946	0.248	0.104	0.310	0.194	0.238

Table 3.3. Summary of pair-wise comparisons of the individual whole blood cytokine responses elicited by *S. haematobium* cercariae (CAP), adult worm (WWH) and egg-specific (SEA) antigens in a naturally exposed human population. CAP-specific innate inflammatory (TNFα and IL-6), Th1 (IFNγ and IL-12p70) and Th17-type (IL-23) responses are higher than those elicited by WWH or SEA. CAP-specific IL-8 and IL-10 levels are higher than those elicited by WWH. SEA-specific IL-2, IL-4, IL-17A and IL-21 levels are higher than those elicited by CAP. SEA-specific IL-6 and IL-8 levels are greater than those elicited by WWH. Ranks of un-transformed antigen-specific cytokine concentrations were compared between antigens via paired Wilcoxon signed rank test. The Z value and 2-tailed asymptotic significance level (Sig.) for each comparison is given (see Figure 3.2-5 for plotted raw data). Values in bold indicate comparisons where $p<0.05$ and values shaded in grey indicate results significant after sequential Bonferroni correction for multiple comparisons.

3.4.2 Phenotypic cytokine responses to *S. haematobium* cercariae, adult worm and egg antigens

Since whole blood cytokine responses occur in the context of multiple and potentially interacting cell types it was important to investigate the phenotypic characteristics of cytokine responses to the different parasite antigens in addition to differences between individual cytokines. To investigate the hypothesis that the *S. haematobium* cercariae, adult worm and egg antigens differ in the cytokine profiles that they elicit, cytokines were grouped according to their patterns of co-variance using factor analysis. Factor analysis extracted 4 significant principal components from the 13 individual cytokine responses assayed in CAP, WWH and SEA-stimulated cultures reflecting the distinct patterns of these responses *in vitro*. Factor loadings for each PC and the cellular phenotype/s with which each is putatively associated are summarised in Table 3.4.

PC1, accounting for the largest percentage of variance in the data (28.8%), is loaded with cytokines associated with innate inflammatory (TNFα, IL-6 and IL-8), Th1 (IFNγ and IL-12p70), Th2 (IL-10) and Th17-type (IL-23) effector responses, corresponding to a mixed inflammatory cytokine profile. PC2 is positively loaded with IL-17A, the characteristic cytokine of Th17-type responses, and IL-2, a T cell derived cytokine which promotes cellular proliferation (chapter 1.5.1). PC3 is loaded with IL-4, IL-5 and IL-10, which are associated with Th2-type immune responses. PC4 is loaded with a combination of Th2-type (IL-13) and Th17-type (IL-21) cytokines.

CAP stimulation resulted in higher PC1 (inflammatory) responses in whole blood cultures relative to all other antigens. Mean factor scores for SEA-suggest that egg antigens induce a moreTh17 (PC2 and PC4) and Th2 (PC3 and PC4)-type cytokine response than CAP. However, these trends were not statistically significant. Mean regression factor scores for each antigen are plotted in Figure 3.6 and results of ANOVA comparison of ANOVA of PC regression factor scores between the 3 antigens are given in Table 3.5.

		Principal Components			
		1	***2***	***3***	***4***
Phenotype	***Cytokine***	**Inflammatory**	**Th17**	**Th2**	**Th2/Th17**
Innate inflammatory	***TNFα***	**0.8**	-0.1	-0.1	0.1
	IL-6	**0.7**	0.2	-0.2	0.1
	IL-8	**0.6**	-0.1	-0.2	-0.2
Th1-type	***IFNγ***	**0.8**	-0.1	0.2	0.0
	IL-2	0.1	**0.9**	-0.2	-0.1
	IL-12p70	**0.8**	-0.2	-0.1	0.1
Th2-type	***IL-4***	0.2	0.3	**0.7**	0.2
	IL-5	0.3	0.4	**0.6**	-0.1
	IL-10	**0.5**	0.0	**0.5**	-0.1
	IL-13	-0.2	0.0	0.2	**0.6**
Th17-type	***IL-17A***	0.1	**0.9**	-0.3	0.0
	IL-21	0.0	0.1	-0.2	**0.8**
	IL-23p19	**0.8**	-0.1	-0.1	0.1
% of total variance		28.8	16.0	11.1	8.9

Table 3.4. *S. haematobium*-specific whole blood cytokine responses group according to distinct immune phenotypes. Table shows PCs 1-4 extracted by regression factor analysis and the factor loadings for each of the square-root(x+1)-transformed *S. haematobium* cercariae, adult worm and egg-specific cytokine variables. Cytokines with factor loadings ≥0.5 or ≤-0.5 for an extracted PC are highlighted in bold. The cellular immune phenotype with which the cytokines are associated is given for each PC. The percentage of total variance in the dataset accounted for by each component is given below the relevant column.

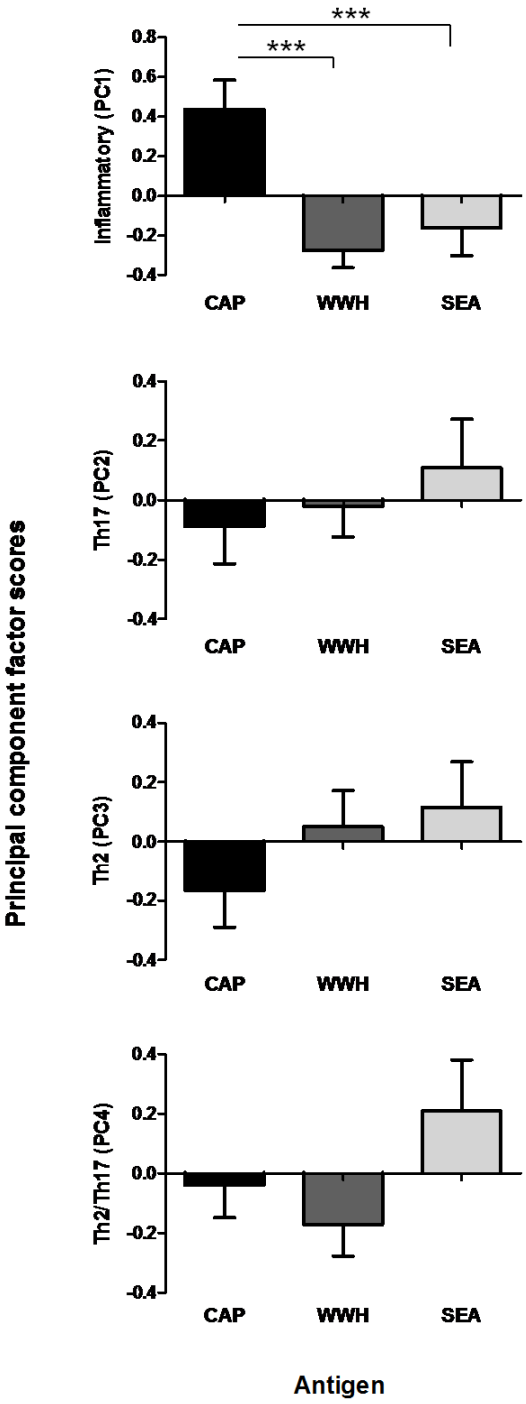


Figure 3.6. *S. haematobium* life cycle stage-specific antigens elicit distinct cytokine profiles. Bar charts shows mean regression factor scores for extracted principal components partitioned by *S. haematobium* antigen (CAP (black), WWH (grey) and SEA (white)). Regression factor loadings were compared between antigens using ANOVA. ***p<0.001. Error bars: standard error of the mean. CAP – cercarial antigen preparation, WWH – whole worm homogenate and SEA - soluble egg antigen

Principal Component	*Phenotype*	*F*	*p*	*Post-hoc comparison*
1	Inflammatory	8.97	**<0.001**	**CAP>WWH & SEA**
2	Th17	0.55	0.580	
3	Th2	1.23	0.295	
4	Th2/Th17	2.11	0.125	

Table 3.5. *S. haematobium* life cycle stage-specific antigens elicit distinct whole blood cytokine phenotypes. Principal component regression factor scores were compared by antigen (CAP, WWH and SEA) using ANOVA (degrees of freedom: 1, 55). Table gives F-statistic and the associated p-value for each extracted component. Significant pair-wise differences between antigen stimulations identified by post-hoc Fisher's least significant difference test are shown. Significant results ($p<0.05$) are highlighted in bold. CAP – cercarial antigen preparation, WWH – whole worm homogenate and SEA - soluble egg antigen

3.5 Discussion

The development of immune responses to schistosome infections is driven by exposure to cercariae, adult worms and egg antigens, which often occur simultaneously in schistosome endemic regions. To-date a number of studies have shown that immune responses to crude antigens prepared from these life-cycle stages are different and used analysis of individual cytokines to infer changes in the immune phenotype as a whole. However, no human studies have directly compared both individual cytokine responses and the cytokine profiles elicited by all 3 schistosome life cycle stages and few have extended cytokine analysis beyond a restricted sub-set of commonly assayed Th1 and Th2 cytokines. Thus, I hypothesised that human whole blood cytokine responses would differ when stimulated *in vitro* with cercariae, adult worm and egg antigens and that Th17 and innate inflammatory-type responses would also vary in response to these 3 life-cycle stages.

The results of this study unequivocally show that *S. haematobium* antigens elicit detectable levels of Th17-type cytokines in whole blood from individuals endemically exposed to infection. IL-17A was present at low but detectable concentrations and parasite-specific IL-21 and IL-23 were readily detectable above background levels present in un-stimulated cultures in most individuals. This extends the observation of a previous study also conducted in a Zimbabwean population, which showed that systemic IL-17A, IL-21 and IL-23 were detectable in plasma samples from an *S. haematobium*-endemic cohort (Milner *et al.* 2010), although in the latter case these observations could not be directly related to parasite antigens. As predicted, Th17-type cytokines were co-produced with a range of other cytokines associated with innate inflammatory, Th1 and Th2-type responses. Interestingly however IL-17A, IL-21 and IL-23 showed distinct patterns of expression in response to the *S. haematobium* antigens, with IL-23 highest in response to CAP, but IL-17A and IL-21 highest in response to SEA. These distinct patterns suggest that IL-17A, IL-21 and IL-23 responses did not follow the expected shared patterns of expression despite their collective association with the Th17 lineage (chapter 1.5.1.4 and 2.3.5.5.1). Although recent human cytokine literature groups these responses together (chapter 2.3.5.5.1), it is important to note that IL-21 was initially characterised as a Th2-type cytokine in mice (Pesce *et al.* 2006; Fröhlich *et al.* 2007) and IL-23 is also known to perpetuate pro-inflammatory/Th1-type cytokine expression by human T cells (Hoeve *et al.* 2006). Thus, whether IL-21 and IL-23 are definitive markers of Th17-type immune responses remains controversial. It is

particularly interesting that IL-21 and IL-13 grouped into a single cytokine profile (PC4) suggesting that *S. haematobium*-specific IL-21 is co-expressed with Th2-type cytokine responses to a greater extent than with Th17-associated IL-17A and IL-23. Both IL-21 and IL-13 appear to play a role in alternative activation of macrophages during experimental schistosomiasis in mice (Pesce *et al.* 2006), although this association has not been investigated in human studies to date.

Direct comparison of the cytokine profiles elicited by the antigens of the 3 life-cycle stages indicated that CAP elicited higher levels of pro-inflammatory effector cytokines than whole homogenised adult worm or egg preparations. CAP elicited the highest concentration of TNFα and IL-6, associated with innate inflammation, IFNγ and IL-12p70, which perpetuate Th1 differentiation and macrophage activation, and IL-23p19, which promotes development of human Th17 cells (chapter 1.5.1). Consistent with the higher levels of pro-inflammatory cytokines by cercariae, CAP also elicited a more pro-inflammatory cytokine profile (PC1) than the other 2 life-cycle stages. Collectively these results suggest that schistosome life-cycle stages affect host immune polarisation differently. There are several potential reasons for the greater cercariae-specific inflammatory responses relative to adult worms or eggs: 1) cercariae express unique antigens that stimulate inflammatory and effector responses, 2) all members of the cohort are primed to respond to cercarial antigens due to repeated exposure, whilst only those who are *S. haematobium* infected are constitutively exposed to egg and adult worm antigens and/or 3) immune responses to adult worm and egg antigens are limited during infection by parasite or host mediated regulatory mechanisms.

The first hypothesis stems from observations that cercariae shed a number of structures, including the acetabular glands, glycocalyx and tail, after penetration of the host (Hansell *et al.* 2008). Thus cercarial homogenates contain a range of antigens (Xu *et al.* 1994; Curwen *et al.* 2004; Hansell *et al.* 2008) that are absent in SEA and WWH. Whilst the reconstituted homogenised cercarial preparations (CAP) used in the current study lack the enzyme activity associated with the secretions of live parasites (Curwen *et al.* 2006; Hansell *et al.* 2008), CAP does contain the carbohydrate antigens (Xu *et al.* 1994) shown to be primarily responsible for parasite-specific IL-6 and IL-12p40 production in murine studies (Jenkins *et al.* 2005a). Cercarial secreted carbohydrate antigens have also been implicated in the induction of TNFα, IL-8, IL-10 and IL-12p40 secretion by human whole blood collected from a Senegalese cohort endemically exposed to both *S. mansoni* and *S. haematobium* (Dr.

Joseph Turner, Liverpool School of Tropical Medicine, U.K., personal communication). It has been previously suggested that somatic antigens shared with adult worm and egg stage parasites (Beisler *et al.* 1984; Jolly *et al.* 2007) found within crude cercarial preparations may 'dilute' inflammatory cytokine responses relative to secreted antigens (Jenkins *et al.* 2005a) and prevent discrimination between life-cycle stage-specific immune responses (Curwen *et al.* 2004). However, the marked difference between the CAP-specific whole blood cytokine profile and those elicited by WWH and SEA suggests that responses to cross-reactive antigens are not immunodominant *in vitro*. In addition, the relative abundance of CAP-specific pro-inflammatory cytokines, particularly those associated with innate inflammation, compared to SEA and WWH-stimulated cultures would be consistent with a more prominent innate effector response to cercariae in human peripheral blood. Although the cell types and numbers expressing these cytokines were not characterised in the current study, work in the murine model of infection has identified antigen presenting cells (APCs), particularly dendritic cells (DCs) and macrophages, as a key source of cercariae-specific inflammatory cytokines *in vivo* (Jenkins *et al.* 2005a; Jenkins and Mountford 2005; Paveley *et al.* 2009). Circulating eosinophils present in human whole blood have also been shown to be a source of adult and egg-stage schistosome-specific TNFα *in vitro* (Silveira-Lemos *et al.* 2008) and cercarial penetration of human skin biopsies leads to an up-regulation of macrophage activation markers (Hansell *et al.* 2008). However, the contribution of circulating innate cell types to the cercariae-specific cytokine response has never been investigated. Thus, an important extension of the current study would be to characterise relative proportions of different cell populations in PBMCs isolated from the same individuals and investigate whether they are related to the profile of whole blood cytokines produced. The latter is an area of on-going study as part of the larger immunoepidemiological field study of Magaya community.

An alternative hypothesis for the less inflammatory cytokine responses elicited by SEA and WWH relative to CAP is that exposure to adult worm and egg antigens are limited relative to cercariae. Exposure to adult worms and eggs may be restricted to cohort members that were patently infected (44.64%) or had experienced fecund infections prior to sampling, whilst all cohort members are at risk of repeated exposure to cercariae during daily water contact activities. In particular young children within the cohort may be naïve to adult worm and egg-specific antigens due to their short history of exposure to infection and this hypothesis is explored in more detail in chapter 4. Evidence for the greater and more constitutive exposure

to cercariae than to adult worms comes from previous observations that cercariae-specific antibodies are present at higher titres than those specific for adult worm antigens (Viana *et al.* 1995) and that larvae-specific IgE titres do not decline after removal of adult schistosomes by chemotherapy (Butterworth *et al.* 1985). Even in infected individuals exposure to adult worm antigens may be limited by sequestration of antigens by live parasites, for example serum antibody responses to many adult worm antigens are only elicited after worms have been killed by praziquantel treatment (Mutapi *et al.* 2003). Thus the proportion of individuals with effector and memory cells primed to secrete cytokines in response to cercarial antigens may exceed those specific for the other life cycle stages. Consistent with the cytokine data presented here, which suggests that immune responses to adult worm antigens are lower or equivalent to those elicited by cercariae and egg-specific antigens, it has been shown that serum antibody responses (IgA, IgM, IgE and IgG1-4) to *S. mansoni* cercariae and egg stage antigens are greater than those to adult worm antigens in an un-treated endemic cohort with a similar age range to the current study (3 – 88 years)(Caldas *et al.* 2000). Equivalent studies of *S. haematobium* cercariae, adult worm and egg-specific antibody responses have not been conducted.

The lower inflammatory responses elicited by WWH and SEA relative to CAP may also result from parasite or host-mediated immunoregulation of cells that recognise these antigens. Chronic schistosomiasis is associated with cellular hypo-responsiveness specific to egg and adult worms (Grogan *et al.* 1998b; Maizels *et al.* 2009) and genomic studies indicate that adult schistosomes express a large number of unique genes with potential immunoregulatory functions, whilst cercariae express genes mainly associated with motility and invasion (Jolly *et al.* 2007). Cytokine responses to egg antigens may also be specifically regulated in infected individuals to limit immunopathology resulting from transmission of eggs to the environment (Wilson *et al.* 2008). However, studies in a variety of tissue-dwelling helminth species suggest that tolerance of patent helminth infection does not inhibit immune responses to *de novo* challenge by larval stage parasites (Smithers and Terry 1967; Day *et al.* 1991a; Day *et al.* 1991b; MacDonald *et al.* 2002). From the data in this chapter it is not possible to infer whether the less pro-inflammatory cytokine profiles elicited by WWH and SEA are due to direct immunoregulation. However, since the same individuals readily produced pro-inflammatory cytokines in parallel MBP-stimulated cultures (data not shown) the low cytokine response to SEA and WWH were not due to non-specific hypo-responsiveness or poor cell viability. Furthermore the marked pro-inflammatory cytokine

profile elicited by cercarial antigens occurred in the context of higher IL-10, as has been observed by others ((Dr. Joseph Turner, Liverpool School of Tropical Medicine, U.K., personal communication), suggesting that CAP-specific IL-10 did not preclude development of a pro-inflammatory cytokine profile. Neutralising antibody studies have shown that adult worm-specific IL-10 inhibits both Th1 and Th2 effector cytokine production during *in vitro* culture of human cells (Grogan *et al.* 1998a; Mutapi *et al.* 2007b), and it would be interesting to conduct similar studies for CAP and SEA-stimulated cultures to investigate whether the regulatory role of IL-10 differs according to life-cycle stage. It is conceivable that cercariae regulate immune responsiveness at the site of infection in the skin (perhaps via the elevated IL-10 responses that their antigens elicit alongside pro-inflammatory responses (Hogg *et al.* 2003b)), but that these mechanisms do not inhibit the cercariae-specific cytokine responses of circulating cells. It should be noted however that infiltrating cells responsible for site-specific regulation of cytokine responses (Cook *et al.* 2011) and active immunomodulatory secretions present in live cercarial challenge in murine models (reviewed by (Jenkins *et al.* 2005b)) are absent from the *in vitro* cultures conducted in this study.

In contrast to CAP, SEA is known to contain specific molecules capable of inducing Th2-type responses, including IL-4 production by human basophils (Schramm *et al.* 2003) and DCs (Everts *et al.* 2009) in purified cell-specific culture. A switch from a Th1 to a Th2-type immune response is also attributed to the onset of egg production in murine schistosomiasis (Pearce *et al.* 1991). Consistent with these observations, SEA elicited more IL-4 than CAP. SEA also had higher mean scores for PC3 (IL-4 and IL-5) and PC4 (IL-13 and IL-21) than CAP, although these differences were not statistically significant. It is also of interest that SEA stimulated cultures had significantly higher IL-17A and IL-21 and a more Th17-type cytokine profile (PC2 (IL-17A and IL-2) and PC4) than CAP, although the latter was not statistically significant. The lack of statistically significant difference between PC2 responses to the 3 life-cycle stages may be partly due to the low number of participants who produced IL-17A at levels detectable above those present in un-stimulated cultures. Th17 cells are relatively uncharacterised in human helminth infections, however their role in chronic autoimmune inflammation and liver disease suggests that they may also contribute to immunopathology in chronic helminthiases (Juszczak and Glabinski 2009; Hammerich *et al.* 2011). The observation that crude cercariae and egg antigen preparations elicit dichotomous cytokine responses (innate inflammatory/Th1-type and Th2/Th17-type respectively (see Table 3.2, Figure 3.2, Figure 3.4) is consistent with expression of distinct antigens by these

life cycle stages and may also reflect differences in exposure patterns during human infection (discussed above).

Despite a number of studies comparing egg and adult worm-specific cytokine responses, there is no consensus in the literature as to their relative immunogenicity in endemically exposed humans. Evidence from whole blood culture studies of human cohorts including both infected and un-infected individuals is conflicting, with some showing that SEA elicits lower IFNγ (Joseph *et al.* 2004a), IL-4, IL-5 and IL-13 (Joseph *et al.* 2004a; Wilson *et al.* 2008) and others showing that SEA elicits higher IL-10 (Joseph *et al.* 2004a), IL-6 and TNFα (Wilson *et al.* 2008) relative to WWH *in vitro*. These inconsistencies may reflect differences between study populations, protocols or simply the omission of specific cytokine assays, which the current study circumvents by covering a comprehensive cytokine panel. The results of this study show that SEA-specific Th1, Th2 and Th17-type cytokine responses do not differ from those elicited by WWH, which may reflect the presence of antigens shared between these parasite life cycle stages as indicated by genetic and serological screens (Beisler *et al.* 1984; Jolly *et al.* 2007). Unlike cercariae, eggs and adult worms are concurrently exposed in venous blood during infection, which may also lead to similarities in the phenotypes and subsets of cells responding to these life cycle stages. It is also possible that SEA and WWH-specific cytokine responses differ below the ELISA detection limit used, although prior optimisation of the standard concentrations for each assay suggests that this is unlikely.

Interestingly, although concerted efforts were made to standardise whole blood culture conditions relative to those of previous studies in *S. mansoni*, the results of the current study contradict observations that WWH elicits higher levels of Th2-type cytokines than SEA (Joseph *et al.* 2004a; Joseph *et al.* 2004b). However, my findings are consistent with *S. haematobium* studies showing that SEA and WWH-specific IL-4 and IL-5 do not differ in the majority of individuals, regardless of whether cultures were conducted for 24, 48 or 72 hours (Scott *et al.* 2000). This may reflect fundamental differences between *S. haematobium* and *S. mansoni* immunobiology (chapter 1.2.3). However, since infection intensities of up to 8,100 eggs/gram of faeces and an infection prevalence of 94% were reported in the *S. mansoni* study (Joseph *et al.* 2004a), significantly greater than that observed for *S. haematobium* in Magaya community, these differences may also reflect distinct exposure patterns within the 2 communities. For example, it seems intuitive that exposure to adult

worm antigens and therefore WWH-specific immune responses would be higher in individuals with high worm burdens and this is evident from studies comparing WWH-specific antibody responses in areas with high and low intensity transmission (Mutapi *et al.* 1997). Thus exposure to parasite antigens may be an important determinant of cytokine polarisation, which I will explore further in chapter 4.

Of the innate inflammatory cytokines measured SEA-stimulated cultures secreted higher IL-6 and IL-8 than WWH, this may reflect the greater immunogenicity of schistosome eggs and/or greater exposure of egg antigens to innate effector cells in vivo. Previous studies have shown that antigens derived from schistosome eggs can elicit innate inflammatory cytokine production by PBMCs via TLR2 ligation (van der Kleij *et al.* 2004) and, consistent with our study, TNFα and IL-6 are also readily produced by *in vitro* culture of human whole blood with S. *mansoni* SEA to a greater extent than WWH (Wilson *et al.* 2008). Although the functional relevance of these differences is unclear, egg-specific inflammatory cytokine responses may contribute to egg-mediated immunopathology as proposed for *S. mansoni* (Wilson *et al.* 2008).

3.6 Conclusions

The findings of this study show for the first time that *S. haematobium* antigens elicit Th17-type cytokines when cultured with whole blood samples from an endemically-exposed community. This is an important step forward for immunoepidemiological field studies of schistosomiasis, where cytokine assays have been biased towards those associated with the Th1 and Th2 lineages.

Direct comparison of whole blood cultures stimulated with crude antigen preparations of *S. haematobium* cercariae, adult worms and eggs also showed that peripheral whole blood cytokine responses differ between the life-cycle stages. These findings are consistent with previous studies showing variation in host responses according to parasite life history (El Ridi *et al.* 1997; Malaquias *et al.* 1997; Webster *et al.* 1997b; Montenegro *et al.* 1999b; Acosta *et al.* 2004), but extend these studies since none investigated immune responses to all 3 life-cycle stages. Furthermore, the current study shows that parasite life-cycle stage affects both the magnitude of individual cytokine responses and the phenotypic characteristics of the

whole blood cytokine profile. In particular, the high levels of pro-inflammatory cytokines elicited by cercarial antigens was in contrast to the low levels of cytokines elicited by adult worms and the predominantly Th2 and Th17 profile of cytokines elevated in response to egg antigens.

The results of this chapter suggest that assaying a broader repertoire of parasite-specific responses provides a greater insight into the complex host-parasite interactions that may shape the immune environment during natural infection. The stage-specific secretion of innate inflammatory and Th17-type cytokines are particularly desirable candidates for further investigation. For example, given the involvement of Th17 in chronic inflammatory diseases in the liver and central nervous system (Juszczak and Glabinski 2009; Hammerich *et al.* 2011) and observations that Th17-type cytokines are associated with morbidity in lymphatic filariasis (Babu *et al.* 2009), it is possible that these responses may also drive immunopathogenic responses to cercariae and eggs in human schistosomiasis.

Chapter 4

The interaction between age, infection intensity and *Schistosoma haematobium-specific* cytokine responses in an endemically-exposed community

4.1 Introduction

The convex relationship between age and schistosome infection intensity in endemic populations suggests that the development of resistance to infection is shaped by changes in age-related factors (Fisher 1934). However, whether this resistance results from development of anti-parasite immunity in response to prolonged exposure to schistosome infection or physiological/behavioural changes with age (or a combination of the two) remains controversial (Fulford *et al.* 1998; Woolhouse 1998). A direct role for the immune response in development of resistance to infection is difficult to discern since parasite-specific immune responses and infection intensity have reciprocal effects and both are influenced by age. This is particularly challenging for permanent residents of schistosome-endemic areas since these individuals are repeatedly exposed to infection throughout their lives and thus age can be considered a proxy of exposure history (Woolhouse 1992, 1994, 1998).

The aim of this chapter is to investigate how the relationship between parasite-specific cytokine responses and schistosome infection intensity is affected by host age in people with life-long exposure to *S. haematobium* in an area of stable, endemic transmission. Firstly I investigate if and how cytokine responses vary with age as an indicator of how age-related factors contribute to the development of distinct schistosome-specific cytokine profiles. Secondly I characterise how correlations between infection intensity and schistosome-specific cytokine responses change with age. The latter is of particular interest since these changes may contribute to the age-related switch from a susceptible phenotype in young children, in whom infection intensity is increasing with age, to a resistant phenotype in adults, in whom infection intensity has declined relative to peak intensities in adolescence (chapter 2.3.7). My study builds upon previous immunoepidemiological surveys showing

that variations in cytokine responses coincide with epidemiological patterns of schistosome infection (Scott *et al.* 2001; Joseph *et al.* 2004a; Mutapi *et al.* 2007b; Milner *et al.* 2010) and extends them by including previously un-characterised cytokines reflecting recent advances in T cell immunology (Diaz and Allen 2007; Allen and Maizels 2011).

I have focused on cytokine responses to adult worm and egg-stage antigens, to which infected hosts are simultaneously exposed, and to the purified schistosome antigen and vaccine candidate glutathionine-S-transferase (GST). Despite promising results from GST-based vaccine trials in animals (chapter 1.8.2), there is conflicting evidence for the contribution of GST-specific antibody response to resistance to *S. mansoni* infection (Correa-Oliveira *et al.* 1989; Grzych *et al.* 1993) and only a single published study to-date has investigated the cytokine responses of endemically-exposed humans to *S. haematobium* GST (Remoué *et al.* 2001). Thus, the findings of this chapter are of relevance both to understanding how parasite-specific cytokine responses develop in an endemic setting and assessing whether GST-specific cytokines contribute to naturally acquired resistance to high intensity infections.

4.2 Hypotheses

- *S. haematobium* adult worm and egg-specific and GST-specific whole blood cytokine profiles vary with age

- Variation in infection intensity is related to parasite-specific cytokine profiles

- The relationship between infection intensity and schistosome-specific cytokine responses differs according to participant age

4.3 Materials and Methods

4.3.1 Study participants

Participants were selected from the cross-sectional cohort of individuals recruited from Magaya community according to the following criteria: 1) provided adequate urine samples for an accurate quantification of *S. haematobium* infection intensity (minimum of 2 samples collected on consecutive days), 2) tested negative for *S. mansoni*, soil-transmitted helminth (STH) and malaria infection, which may influence immune responses to *S. haematobium* antigens (see chapter 1.4.5 and 2.3.6), 3) had not received prior treatment for schistosomiasis, which may artificially increase exposure to parasite antigens and thus affect age and exposure related patterns of cytokine responses (see chapter 1.7.3) and 4) were long-term permanent residents of the area. In the latter case questionnaire-based assessment of residential history (Appendix 1) allowed individuals who had recently moved to the area from urban and schistosome non-endemic areas or those who were visitors to the study site to be excluded. It was considered important to exclude individuals on this basis so that host age could be used as a proxy for duration of exposure to schistosome infection in the area ('exposure history'). Furthermore, local transmission intensity is an important determinant of the rate at which resistance to high intensity infections (Hagan 1992; Woolhouse 1998) and parasite-specific immune responses (Mutapi *et al.* 1997) develop. Exclusion of recent immigrants to Magaya did not significantly affect the mean age or male: female ratio of the selected cohort relative to that of the cross-sectional cohort of Magaya community as a whole (chapter 2.3.7).

198 individuals met the above selection criteria and are included in the current study. Of these 15 tested positive for HIV infection. Potential variations in schistosome-specific cytokine responses due to HIV infection were accounted for in all statistical analyses.

4.3.2 Immunological assays

As described in chapter 2.3.5.5 and outlined in chapter 3, all participants provided a whole blood sample which was stimulated for 48 hours at 37°C with 10µg/ml crude preparations of

S. haematobium adult worms (WWH) and eggs (SEA) in parallel cultures. Purified GST was used at a concentration of 2μg/ml. Culture conditions for crude and purified antigen stimulations were identical in all other respects and were conducted using the same diluted whole blood sample stock for each individual. Cultures stimulated with MBP (2μg/ml) acted as a positive control, confirming that cultured cells were viable and capable of producing cytokines in response to antigen stimulus (mean MBP-specific cytokine responses reported in Appendix 2). Un-stimulated cultures acted as negative controls for levels of spontaneous cytokine production (mean cytokine concentrations present in un-stimulated cultures reported in Appendix 2).

Cytokines associated with innate inflammatory (TNFα, IL-6 and IL-8), Th1 (IFNγ, IL-2 and IL-12p70), Th2 (IL-4, IL-5, IL-10 and IL-13) and Th17 (IL-17A, IL-21 and IL-23p19) responses were assayed in culture supernatants by ELISA and a mean concentration (ng/ml) obtained from duplicate ELISA wells.

4.3.3 Statistical Analyses

Epidemiological patterns of *S. haematobium* infection intensity and prevalence were characterised within all members of Magaya community who provided blood samples (n = 284) and used to identify the age range in which infection intensity was increasing (age 5-10 years, n = 109), peaking (age 11-12 years, n = 60) and declining (age 13-84 years, n = 111). Categorising age allowed the non-linear relationship between infection intensity and age to be incorporated into ANOVA models (Mutapi and Roddam 2002) and has been effectively used in previous studies of human schistosomiasis (Mutapi *et al.* 2008; Milner *et al.* 2010). The selection of these age groups is described in detail in chapter 2.3.7.

To verify that the age-infection intensity dynamics of the selected cohort reflected those of all recruits from Magaya community log10(x+1) transformed *S. haematobium* egg counts/10ml urine (infection intensity) were compared between the 3 age groups via univariate ANOVA with age group as the explanatory variable. Analysis was conducted on log10(x+1)-transformed data since raw egg counts exhibited a strong negative binomial distribution and unequal variance between age groups (Mutapi and Roddam 2002). Exploratory analysis to check that transformed data met the assumptions of parametric tests indicated that the residuals of log10(x+1)-transformed infection intensity data from ANOVA

models were normally distributed and the variances of the 3 age groups were equal. Gender-specific variations in water contact behaviour described in previous studies (Chandiwana and Woolhouse 1991; de Moira *et al.* 2010) and the effect of HIV status were accounted for by including sex and HIV status prior to age group in the sequential sum of squares ANOVA (Mutapi and Roddam 2002). To verify that variations in the mean infection intensity of the 3 age groups were not due to age-dependent variations in the frequency of water contacts, un-transformed mean daily frequencies of contact with local water sources (assessed by a water contact survey in a sub-set of individuals included in the cohort (n = 76)) were also compared between the 3 age groups by ANOVA.

To characterise distinct cytokine profiles within antigen-stimulated whole blood culture supernatants, square root(x+1) transformed WWH and SEA-specific whole blood cytokine responses were grouped by factor analysis into a smaller number of principal components (PCs) (Sokal and Rohlf 1995c). All WWH and SEA-specific cytokine responses were included as separate factors in a single factor analysis since adult worms and eggs are present simultaneously during infection. Thus the extracted principal components (PCs) reflect both the antigen specificity (WWH and/or SEA) and groups of cytokines (cytokine profiles) that account for variation between participants. Extracted PCs were included in subsequent analyses only if they: 1) accounted for a greater than average proportion of variance in the original cytokine data (eigenvalue >1) (Kaiser 1960) and 2) correlated with at least 2 of the original cytokine variables with a factor loading of greater than 0.5 or less than -0.5. The cytokine profile of each PC was determined according to the putative association of cytokine responses with innate inflammatory, Th1, Th2, Th17, pro-inflammatory and regulatory immune phenotypes (chapter 2.3.5.5.1). A separate factor analysis was conducted for GST-specific cytokine responses. Full details of the factor analysis procedure are given in chapter 2.5.3.

To address the hypothesis that the parasite-specific cytokine profiles identified by factor analysis were influenced by age ANOVA was used. PC regression factor scores were the dependent variables and sex (male, female), HIV status (positive, negative), log10(x+1)-transformed infection intensity (covariate), age group (5-10, 11-12 and 13+ years) and the infection intensity-age group interaction were included as explanatory variables. Sequential sums of squares were used for the analysis to allow for the fact that infection prevalence was higher in males than in females (as has been observed in previous studies of schistosome

infection in Zimbabwe (Mutapi *et al.* 2007b)), the potential for HIV infection to affect exposure to schistosome antigens (Karanja *et al.* 1997) and to account for variation in the cytokine profiles due to infection intensity prior to comparing cytokine responses between the 3 age groups. The infection intensity-age group interaction term was included to assess whether differences between age groups were dependent on the intensity of infection within these groups. PC regression factor scores and the residuals of ANOVA of PC factor scores met the assumptions of parametric tests. Post-hoc pair-wise comparisons between the cytokine profiles of the 3 age groups were conducted using Fisher's least significant difference test. The test involves calculating the square-root of the residual mean squares from the ANOVA for the 2 groups being compared and dividing the difference between the 2 square roots by the standard error of that difference to give a t-ratio (T) and its associated p-value (Sokal and Rohlf 1995b), which are reported where relevant.

To address the hypothesis that variation in infection intensity was related to parasite-specific cytokine profiles, I used a univariate ANOVA with log10(x+1)-transformed infection intensity as the dependent variable and sex, HIV status, age group and cytokine profile (principal component regression factor scores) as explanatory variables. Variation due to all other explanatory variables was accounted for prior to age group by using sequential sums of squares. The interaction between age group and each principal component was also included to test the hypothesis that the relationship between infection intensity and cytokine responses was age-dependent. Exploratory analysis indicated that the transformed infection intensity data and residuals of ANOVA met parametric assumptions. Preliminary analysis also indicated that the effect of each cytokine profile was influenced by the order in which it was entered into the model, indicating that their independent effects on infection intensity are more appropriately characterised in separate univariate ANOVA rather than a single MANOVA (Mutapi and Roddam 2002). Post-hoc Pearson's 2-tailed correlation analysis was used to identify the direction of the relationship between residual factor scores for each cytokine profile from the ANOVA and residuals of infection intensity (after accounting for sex, HIV status and age).

The sequential Bonferroni-adjusted significance level is reported in addition to raw p-values for all tests (main effects, interactions and post-hoc tests) to provide an indicator of the significance of the relationship after accounting for multiple comparisons (Holm 1979; Rice 1989).

4.4 Results

4.4.1 Infection intensity and prevalence

The cross-sectional distribution of infection and prevalence by age provides an indicator of how levels of infection may change across the life-span of an individual, permanent resident of Magaya community. It is also important to identify other potential reasons for variation in infection levels, including sex (since hormones and certain water contact behaviours may be gender-specific) and HIV status (which may increase susceptibility to infection). *S. haematobium* infection intensities within the cohort were significantly higher in males than females ($F_{1, 190}$: 12.23, p = 0.001) and differed significantly between the age groups ($F_{2, 190}$: 10.30, p < 0.001), consistent with the age-infection distribution in all recruits from Magaya community (see chapter 2.3.7). Children aged 11-12 years had higher mean infection intensities than younger children (5-10 years, T: 3.53, p = 0.001) and older children and adults (13+ years, T: 4.54, p<0.001). Infection intensity was not significantly affected by HIV status ($F_{1, 190}$: 0.846, p = 0.359). The distribution of *S. haematobium* infection intensity by age group is shown in Figure 4.1 and the demographic and infection characteristics of the study cohort are summarised in Table 4.1.

Of the individuals who provided details of their daily water contact activities (n = 76), mean daily water contacts did not significantly differ according to age group ($F_{2,72}$: 2.862, p = 0.064) and the effect of age group on infection intensity was significant after accounting for mean daily water contacts in these individuals ($F_{2,71}$: 4.856, p = 0.011). Together these observations indicate that the convex age-infection distribution does not result from age-dependent variation in water contact behaviour alone.

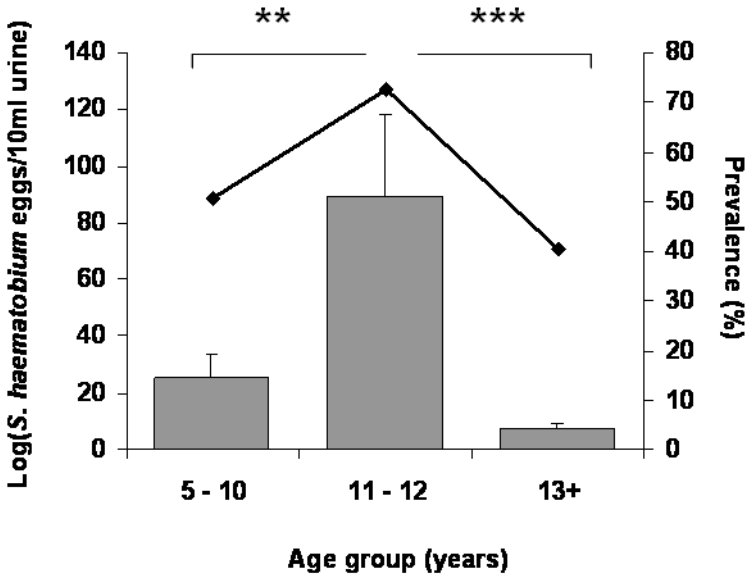


Figure 4.1. Pre-treatment *S. haematobium* infection distribution by age group. Bar chart indicates the mean log10(x+1)-transformed infection intensity of each age group and the black line indicates percentage prevalence of infection for each age group. Error bars: standard error of the mean. Mean infection intensity was compared between age groups by univariate ANOVA. ** p <0.01, ***p<0.001.

	Age group (years)		
	5 - 10	***11 – 12***	***13+***
n	83	33	82
Mean age	7.4	11.6	28.3
Number of males: females	43:40	18:15	33:49
HIV +ve	4	2	9
Mean infection intensity*(S.E.M.)	25.23 (8.37)	89.14 (29.38)	7.20 (1.94)
Infection range*	0 - 481	0 – 692	0 - 107
Infection prevalence (%)	50.6	72.7	40.2

Table 4.1. Demographic and *S. haematobium* infection characteristics of each age group. *Infection intensity quantified as arithmetic mean egg counts/10ml urine. S.E.M. – standard error of the mean.

4.4.2 Adult worm and egg-specific cytokine profiles

Since adult worm and egg antigens are simultaneously exposed to circulating blood cells during infection I sought to characterise the cytokine profiles elicited by these antigens *in vitro*. Factor analysis of all SEA and WWH-specific cytokines extracted 6 principal components reflecting inflammatory (PC1: TNFα, IL-6, IFNγ, IL-12p70 and IL-23), regulatory/Th17 (PC2: IL-10 and IL-21), Th2/Th17 (PC3: IL-13 and IL-21), IL-4/IL-13 (PC4) , Th2/regulatory (PC5: IL-5 and IL-10) and Th17 (PC6: IL-17A)-type cytokine profiles. IL-4 was positively loaded and IL-13 was negatively loaded onto PC4 meaning that individuals with high PC4 factor scores have a more IL-4 polarised response and relatively less prominent IL-13 response. In most cases cytokines specific for both adult worm and egg antigens had the same association (positive/negative) and similar values for factor loadings onto each extracted principal component and were grouped together, supporting the similarities between WWH and SEA-specific cytokine profiles identified in chapter 3. The only exceptions were TNFα and IL-5, for which egg, but not adult worm-specific responses grouped with inflammatory cytokine responses (PC1) and Th2/regulatory (PC6) responses respectively. The factor analysis and extracted components are summarised in Table 4.2. The mean and standard error of the mean for individual SEA and WWH-specific cytokine responses within the cohort (n = 198) is given in Appendix 2.

			Principal Components					
			1	***2***	***3***	***4***	***5***	***6***
Phenotype	***Cytokine***	***Antigen***	**Inflammatory**	**Regulatory /Th17**	**Th2/ Th17**	**IL-4/ IL-13**	**Th2/ Regulatory**	**Th17**
Innate Inflammatory	***TNFα***	***SEA***	**0.5**	-0.2	0.0	0.4	0.4	-0.3
		WWH	0.2	0.1	-0.2	-0.1	-0.1	0.2
	IL-6	***SEA***	**0.5**	0.1	-0.2	0.1	0.1	-0.3
		WWH	**0.5**	0.4	-0.3	-0.1	0.0	0.1
	IL-8	***SEA***	0.2	0.2	0.1	0.0	-0.3	-0.4
		WWH	0.4	0.3	0.3	-0.1	-0.4	-0.3
Th1-type	***IFNγ***	***SEA***	**0.7**	-0.1	0.2	0.0	0.2	0.1
		WWH	**0.6**	0.0	0.1	-0.2	-0.1	0.3
	IL-2	***SEA***	-0.1	-0.1	0.0	0.0	0.0	0.1
		WWH	0.0	0.0	0.2	-0.2	-0.1	0.0
	IL-12p70	***SEA***	**0.8**	-0.1	0.0	-0.1	0.0	0.2
		WWH	**0.6**	-0.1	0.0	-0.2	-0.1	0.2
Th2-type	***IL-4***	***SEA***	0.0	0.0	0.3	**0.5**	0.0	0.4
		WWH	0.1	0.0	0.2	**0.5**	0.0	0.4
	IL-5	***SEA***	0.0	-0.3	-0.1	0.2	**0.5**	-0.2
		WWH	0.0	-0.3	-0.1	0.1	0.4	-0.1
	IL-10	***SEA***	0.1	**0.7**	-0.4	-0.1	**0.5**	0.0
		WWH	0.0	**0.6**	-0.3	-0.1	**0.5**	0.1
	IL-13	***SEA***	-0.1	-0.1	**0.5**	**-0.6**	0.4	0.0
		WWH	-0.1	-0.1	**0.6**	**-0.6**	0.3	0.0
Th17-type	***IL-17A***	***SEA***	0.2	0.0	0.1	0.0	0.2	**0.5**
		WWH	0.0	0.0	0.2	0.1	0.2	**0.5**
	IL-21	***SEA***	0.0	**0.5**	**0.6**	0.3	0.0	-0.2
		WWH	0.0	**0.6**	**0.5**	0.3	0.1	-0.1
	IL-23p19	***SEA***	**0.6**	-0.2	0.0	0.2	0.2	-0.4
		WWH	**0.6**	0.1	-0.1	-0.1	-0.3	0.0
% of total variance			14.2	8.0	7.4	7.3	6.7	6.5

Table 4.2. Whole blood cytokine profiles grouped by factor analysis of all adult worm and egg-specific cytokine responses. Table shows PCs1-6 extracted by regression factor analysis and the factor loadings for each of the square-root(x+1)-transformed whole worm homogenate (WWH) and soluble egg antigen (SEA)-specific cytokine variables. Cytokines with factor loadings ≥0.5 or ≤-0.5 for an extracted PC are highlighted in bold. The cellular immune phenotype with which the cytokines are associated is given for each PC. The percentage of total variance in the dataset accounted for by each PC is given below the relevant column.

4.4.3 Variation in adult worm and egg-specific cytokine profiles by age group

Having identified the major patterns of adult worm and egg-specific cytokine responses I investigated the hypothesis that these the cytokine profiles extracted by factor analysis varied according to age group after accounting for variation due to sex, HIV status and infection intensity. Although mean factor scores for adult worm and egg-specific inflammatory responses (PC1) were highest in 11-12 year olds and scores for Th2/Th17 (PC3), IL-4/ IL-13 (PC4) and Th2/regulatory (PC5) responses were lowest in 11-12 year olds, age group was only a significant explanatory variable for regulatory/Th17 (PC2) and Th17 (PC6)-type responses. Pair-wise comparisons confirmed that 5-10 year olds had higher scores for regulatory/Th17-type responses than 11-12 (T: 2.41, $p = 0.017$) and 13+ year olds (T: 3.32, $p = 0.001$). Th17-type responses (PC6) were significantly lower in 13+ year olds than 5-10 (T: 3.31, $p = 0.001$) or 11-12 year olds (T: 3.43, $p = 0.001$). In addition, regulatory/Th17-type responses to adult worm and egg antigens were significantly influenced both by current infection intensity and by the interaction between age group and infection intensity, indicating that the effects of age and infection on the schistosome-specific cytokine environment are inter-dependent. Mean scores are plotted by age group for each of the cytokine profile in Figure 4.2 and results of statistical analyses are summarised in Table 4.3.

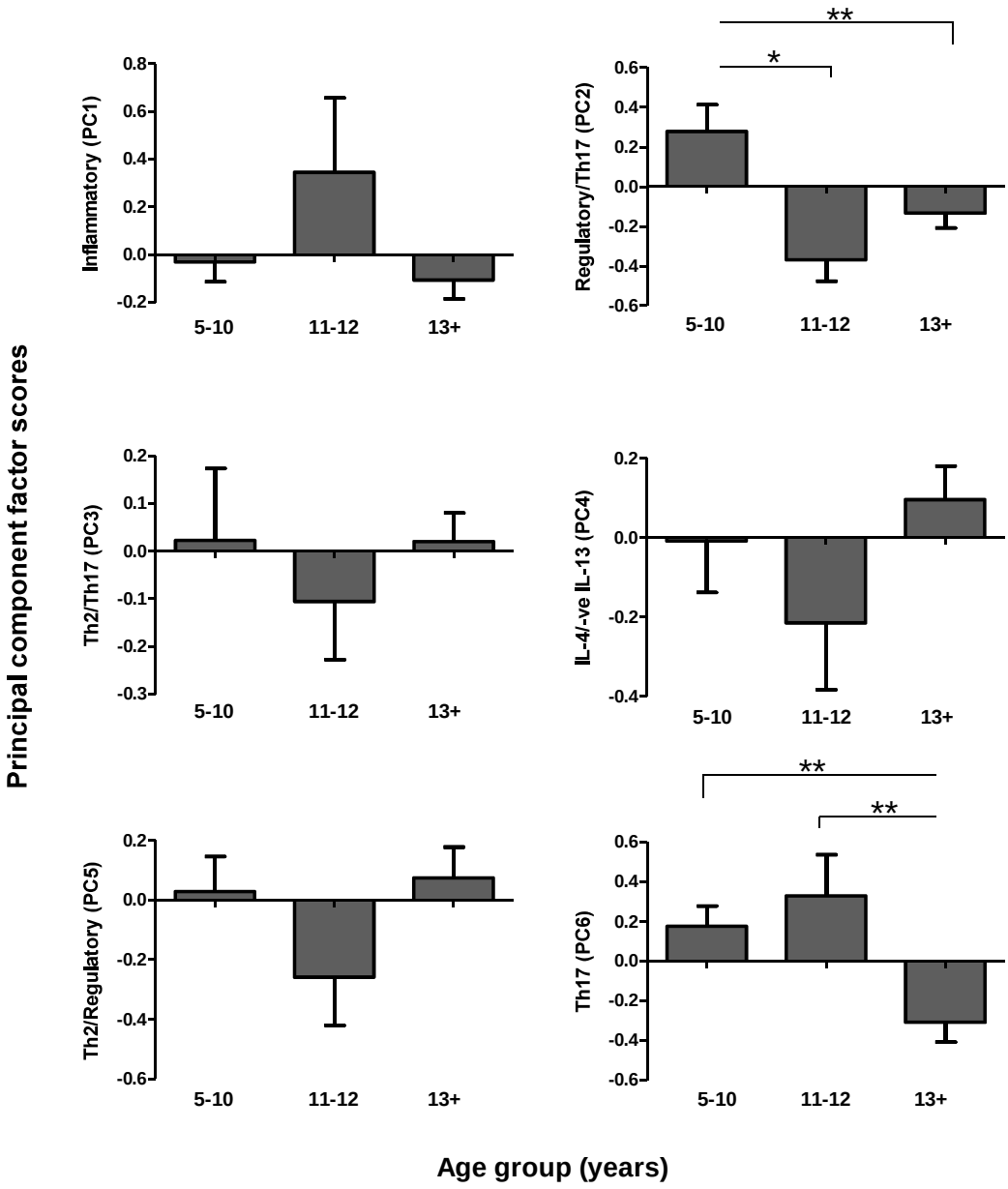


Figure 4.2. Adult worm and egg-specific cytokine profiles differ between age groups. Bar charts represent the variation in mean adult worm and egg-specific principal component regression factor scores for each age group after accounting for variation due to sex, HIV status and infection intensity in ANOVA. Error bars: standard error of the mean, *p<0.05, **p<0.01.

Factor *(degrees of freedom)*	*Inflammatory (PC1)*		*Regulatory/Th17 (PC2)*		*Th2/Th17 (PC3)*		*IL-4/IL-13 (PC4)*		*Th2/Regulatory (PC5)*		*Th17 (PC6)*	
	F	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***P***
Sex *(1,190)*	0.058	0.810	4.522	**0.035** **females>males**	2.188	0.141	0.039	0.844	0.022	0.882	0.502	0.479
HIV status *(1,190)*	0.015	0.904	2.258	0.135	0.452	0.502	1.172	0.280	2.396	0.123	0.037	0.848
Infection intensity *(1,190)*	0.163	0.686	6.099	**0.014** **+ve correlation**	0.000	0.988	1.903	0.169	0.044	0.834	1.678	0.197
Age group *(2,190)*	2.750	0.067	7.350	**0.001** **5-10>11-12** **5-10> 13+**	0.240	0.787	1.276	0.282	1.520	0.220	8.060	**<0.001** **13+<5-10** **13+<11-12**
Age group* ***Infection intensity*** *(2,190)*	0.36	0.7	5.44	**0.005**	1.25	0.3	0.736	0.481	0.086	0.918	0.263	0.769

Table 4.3. Adult worm and egg-specific 'Regulatory/Th17' and 'Th17'-type cytokine responses are influenced by host age group and the interaction between age group and infection intensity. Variations due to sex, HIV status and infection intensity (log10(x+1)-transformed mean *S. haematobium* egg counts/10ml urine) alone were accounted for before age group and the interaction between age group and infection intensity by using a sequential sums of squares ANOVA. Significant results ($p<0.05$) are highlighted in bold and those significant after sequential Bonferroni correction for multiple comparisons are shaded grey.

4.4.4 Relationship between infection intensity and adult worm and egg-specific cytokine profiles

Since the effect of age and infection intensity on schistosome-specific cytokine profiles were inter-dependent I hypothesised that the relationship between infection levels and schistosome-specific cytokine profiles would vary with age. Whilst the latter may be inferred from the results described in 4.4.3, it has been previously suggested that age alone is sufficient to explain the distribution of infection intensity (chapter 1.2.4) and thus it was important to account for the effects of age group prior to those of participant cytokine profiles in the analysis.

Consistent with the significant association between cytokine profiles and infection intensity (4.4.3) variation in infection intensity within the cohort was related to variation in parasite-specific whole blood cytokine responses after accounting for the effects of sex, HIV status and age group (Table 4.4). Post-hoc Pearson's 2-tailed correlation analysis confirmed a significant positive relationship between infection intensity and regulatory/Th17-type responses (PC2) suggesting that individuals with high intensity infections have a more regulatory/Th17-polarised cytokine profile than un-infected individuals or those with light infections. Infection intensity was negatively correlated with Th17-type responses (PC6). The relationship between adult worm and egg-specific cytokine profiles and infection intensity are shown in Figure 4.3 and the direction and significance of the correlation between the 2 variables is indicated for each plot.

In addition to the effects of age group and regulatory/Th17-type cytokine responses, infection intensity was also influenced by the interaction between these two variables (not significant after Bonferroni correction). Whilst there was a significant positive correlation between mean *S. haematobium* egg counts and regulatory/Th17 factor scores in the youngest age group (5-10 years), there was no correlation in either of the two older age groups. The correlation between infection intensity and regulatory/Th17 factor scores within the different age groups is shown in Figure 4.4. Infection intensity was not significantly influenced by the interaction between age group and any of the other adult worm and egg-specific principal components.

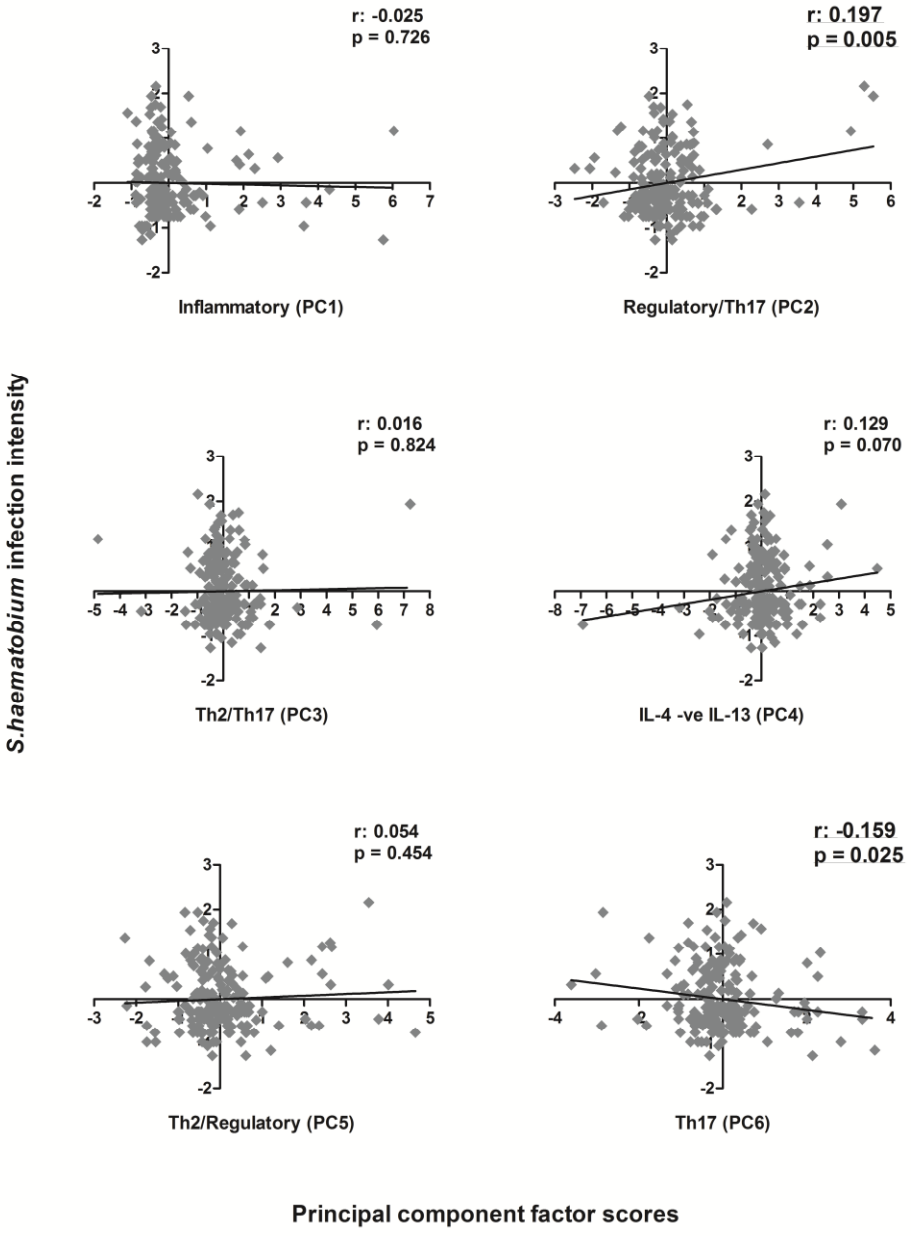


Figure 4.3. Correlations between infection intensity and adult worm and egg-specific whole blood cytokine profiles. Residual variance in log10(x+1)-transformed infection intensity after accounting for sex, HIV status and age group is plotted against residual variance in participant factor scores for each cytokine profile (PC) identified by factor analysis. The Pearson's r value and significance level of correlations are indicated for each plot and significant correlations are highlighted in bold and underlined. Black lines: best fit values from linear regression. –ve IL-13 - IL-13 is negatively associated with PC4 and therefore higher PC4 factor scores correspond to a lesser change in IL-13 responses.

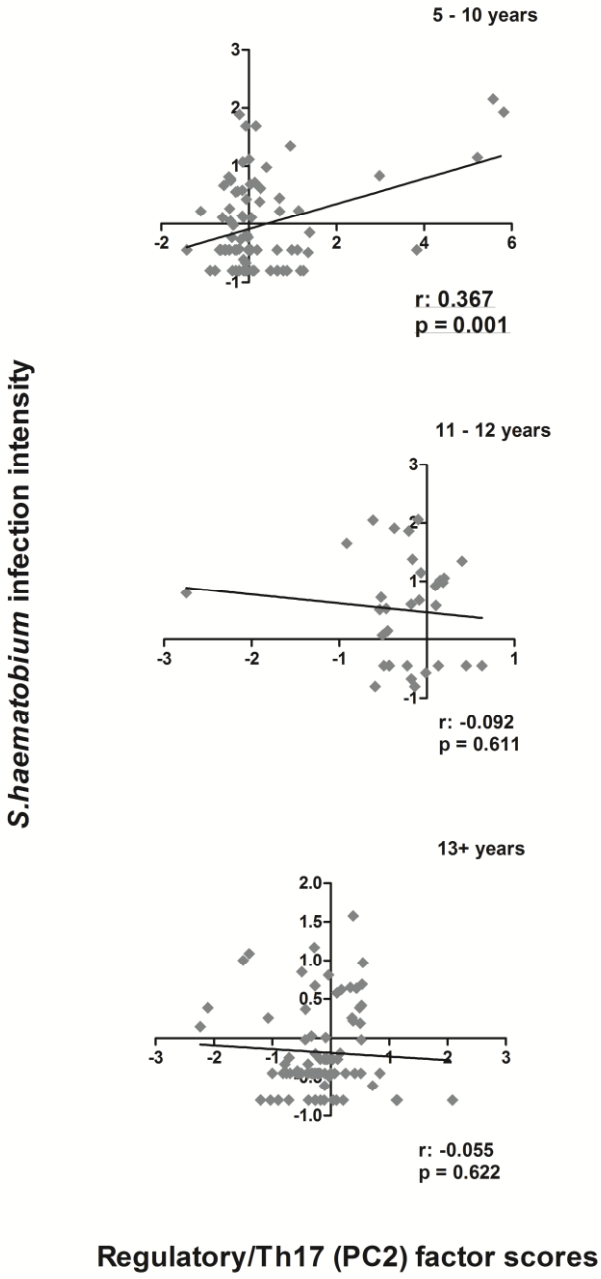


Figure 4.4. Schistosome infection intensity is influenced by the interaction between an adult worm and egg-specific regulatory/Th17-polarised cytokine profile and age group. Residual variance in log10(x+1)-transformed infection intensity after accounting for sex and HIV status is plotted against residual variance in regulatory/Th17 (PC2) factor scores for each of the 3 age groups. The Pearson's r value and significance level of correlations are indicated for each plot and significant correlations are highlighted in bold and underlined. Black lines: best fit values from linear regression.

Factor *(degrees of freedom)*	***Infection intensity***		
	F	***p***	***Post-hoc Comparisons***
Sex *(1, 190)*	12.229	**0.001**	**males > females**
HIV status *(1, 190)*	0.846	0.359	
Age group *(2, 190)*	10.304	**<0.001**	**11-12 > 5-10 years** **11-12 > 13+ years**
WWH & SEA-specific cytokine profiles:			
PC1 (Inflammatory) *(1, 190)*	2.750	0.067	
PC1*Age group *(2, 190)*	0.36	0.7	
PC2 (Regulatory/Th17) *(1, 190)*	**7.350**	**0.001**	**+ve correlation**
PC2*Age group *(2, 190)*	**5.44**	**0.005**	
PC3 (Th2/Th17) *(1, 190)*	0.240	0.787	
PC3*Age group *(2, 190)*	1.25	0.3	
PC4 (IL-4/IL-13) *(1, 190)*	1.276	0.282	
PC4*Age group *(2, 190)*	0.736	0.481	
PC5 (Th2/Regulatory) *(1, 190)*	1.520	0.220	
PC5*Age group *(2, 190)*	0.086	0.918	
PC6 (Th17) *(1, 190)*	**8.060**	**<0.001**	**-ve correlation**
PC6*Age group *(2, 190)*	0.263	0.769	

Table 4.4. Variation in infection intensity is associated with adult worm and egg-specific cytokine profiles. Results of ANOVA of log10(x+1)-transformed mean *S. haematobium* egg counts/10ml for each cytokine profile (PC) using sequential sums of squares to account for variation due to sex, HIV status and age group. The effects of each PC was analysed in separate ANOVA. Significant results ($p<0.05$) are highlighted in bold and those significant at the sequential Bonferroni-adjusted significance level are shaded grey. +ve – positive, -ve – negative

4.4.5 GST-specific cytokine profiles

The major cytokine profiles elicited by GST *in vitro* were identified by factor analysis, which extracted 5 PCs. As for adult worm and egg-specific cytokine profiles, GST-specific inflammatory (IL-6, IFNγ, IL-12p70 and IL-23) and regulatory/Th17 (IL-10 and IL-21)-type responses were grouped into distinct PCs (PC1 and PC3 respectively). PCs reflecting a combination of IL-2, IL-8 and IL-13 responses (PC2), Th2/Th17 (PC4) and Th2 (PC5)-type cytokine profiles were also extracted. GST-specific cytokine profiles identified by the factor analysis are summarised in Table 4.5. . The mean and standard error of the mean for individual GST-specific cytokine responses within the cohort is given in Appendix 2.

			Principal Components				
			1	***2***	***3***	***4***	***5***
Phenotype	***Cytokine***	***Antigen***	**Inflammatory**	**IL-2/IL-8 /IL-13**	**Regulatory /Th17**	**Th2/ Th17**	**Th2**
Innate Inflammatory	***TNFα***	***GST***	0.4	0.1	0	0	0
	IL-6	***GST***	**0.7**	0.1	0.2	0.1	-0.4
	IL-8	***GST***	0.4	**0.5**	0.2	0	-0.4
Th1-type	***IFNγ***	***GST***	**0.8**	-0.3	-0.1	-0.1	0.3
	IL-2	***GST***	0.1	**0.7**	0.2	0.2	0.2
	IL-12p70	***GST***	**0.8**	-0.3	-0.1	-0.1	0.2
Th2-type	***IL-4***	***GST***	0	-0.2	-0.1	**0.7**	0.1
	IL-5	***GST***	-0.1	-0.1	0	0.2	**0.5**
	IL-10	***GST***	-0.1	-0.3	**0.8**	0.2	0.1
	IL-13	***GST***	0	**0.6**	0.1	0.1	**0.5**
Th17-type	***IL-17A***	***GST***	0	-0.1	-0.2	**0.7**	-0.2
	IL-21	***GST***	-0.1	-0.3	**0.8**	0	0
	IL-23p19	***GST***	**0.7**	0	0	0	0.1
% of total variance			20.5	11.9	10.6	9.4	8.6

Table 4.5. Whole blood cytokine profiles grouped by factor analysis of all GST-specific cytokine responses. Table shows PCs 1-5 extracted by factor analysis and the factor loadings for each of the square-root(x+1)-transformed *S. haematobium* GST-specific cytokines. Cytokines with loadings ≥0.5 or ≤-0.5 are highlighted in bold for each PC. The immune phenotype with which cytokines are associated is given for each PC. The percentage of total cytokine variance accounted for by each PC is given below each column.

4.4.6 Variation in GST-specific cytokine profiles by age group

As an abundant constituent of the schistosome proteome I hypothesised that GST-specific cytokine profiles, like those elicited by WWH and SEA, would vary with age and this was confirmed by comparison of the mean regression factor scores for the GST-specific cytokine profiles between the 3 age groups. GST-specific inflammatory cytokine responses were highest in 11-12 year olds relative to 5-10 (T: 3.16, p = 0.002) and 13+ year olds (T: 3.31, p = 0.001). As observed for adult worm and egg-specific cytokine responses, GST-specific regulatory/Th17 responses were highest in the youngest age group, but only significantly differed between 5-10 and 11-12 year olds (T:2.41, p = 0.017). None of the other GST-specific cytokine profiles (PC2, PC4 and PC5) significantly differed according to age group. GST-specific cytokine profiles were not significantly influenced by the interaction between age group and infection intensity. Factor scores for GST-specific cytokine profiles are plotted by age group in Figure 4.5 and statistical analyses are summarised in Table 4.6.

4.4.7 Variation in infection intensity according to GST-specific cytokine profiles

Since GST is proposed as an anti-schistosome vaccine I investigated whether infection intensity was related to the GST-specific cytokine profiles after accounting for the effects of age group. Infection intensity was not significantly related to variation in GST-specific cytokine profiles or the interaction between GST-specific cytokine profiles and age group (Figure 4.6). ANOVA results are summarised in Table 4.7.

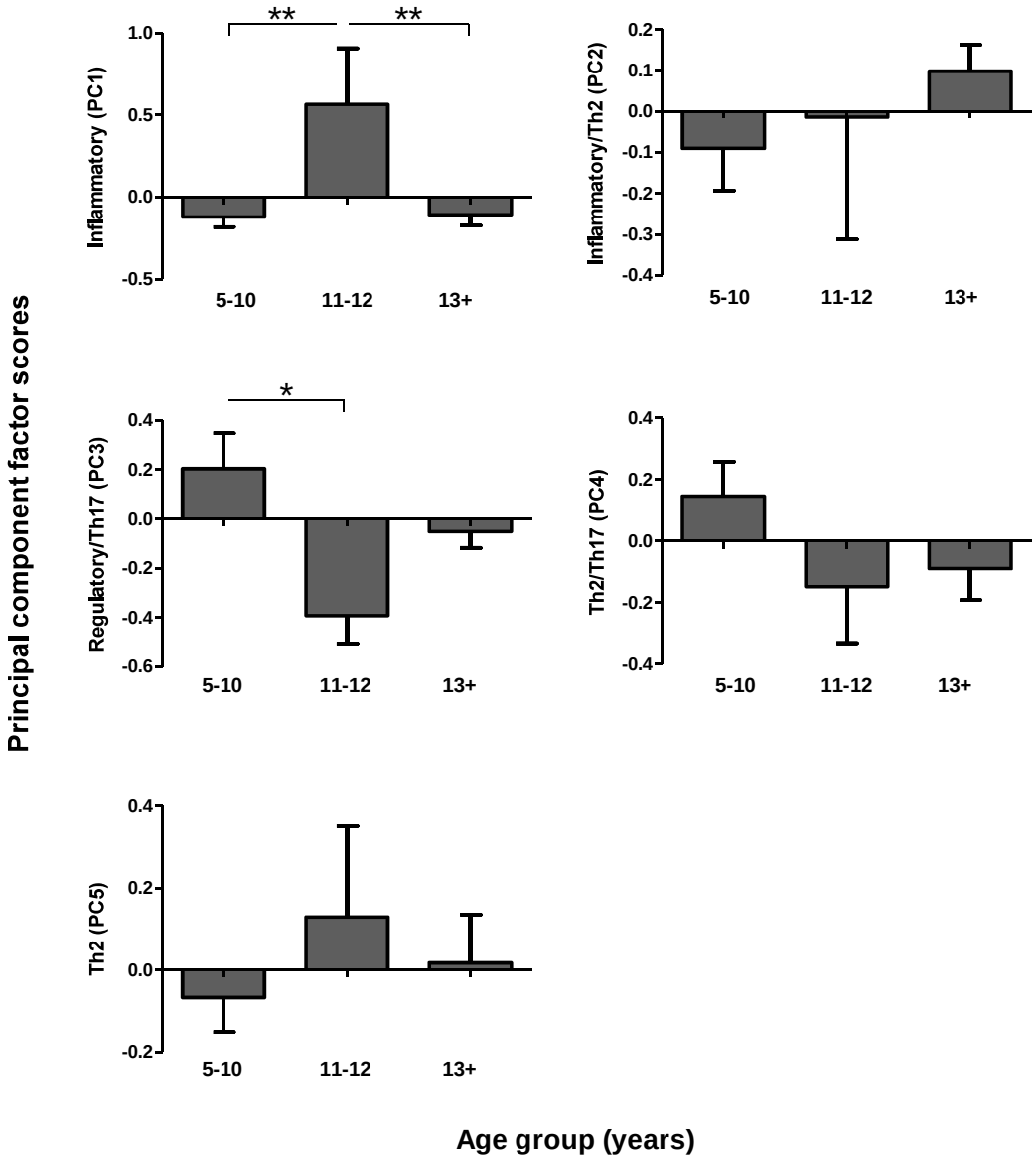


Figure 4.5. GST-specific cytokine profiles differ between age groups. Bar charts represent the residual variation in mean GST-specific PC regression factor scores for each age group after accounting for variation due to sex, HIV status and infection intensity. Error bars: standard error of the mean, *p<0.05, ** p<0.01.

Factor$_{(degrees\ of\ freedom)}$	*Inflammatory (PC1)*		*Inflammatory/Th2 (PC2)*		*Regulatory/Th17 (PC3)*		*Th2/Th17 (PC4)*		*Th2 (PC5)*	
	F	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
Sex$_{(1, 190)}$	2.278	0.133	0.257	0.613	1.968	0.162	0.045	0.833	0.068	0.794
HIV status$_{(1, 190)}$	1.155	0.284	0.566	0.453	1.125	0.290	1.346	0.247	3.772	0.054
Infection intensity$_{(1, 190)}$	1.634	0.203	0.231	0.631	1.090	0.298	2.626	0.107	1.271	0.261
Age group$_{(2, 190)}$	7.503	**0.001** **11-12>5-10** **11-12> 13+**	0.740	0.478	4.817	**0.009** **5-10>11-12**	1.616	0.201	0.507	0.603
Age group* ***Infection intensity***$_{(2, 190)}$	0.859	0.425	0.466	0.628	1.842	0.161	0.707	0.494	1.638	0.197

Table 4.6. GST-specific inflammatory and regulatory/Th17-type cytokine responses are influenced by host age group. Results of ANOVA of PC regression factor scores compared by age group and the interaction between age group and infection intensity. Variation in cytokine profiles due to sex, HIV status and infection intensity (log10(x+1)-transformed *S. haematobium* mean egg counts/10ml urine) was accounted for using sequential sums of squares. Significant results ($p<0.05$) are highlighted in bold and those significant after sequential Bonferroni correction for multiple comparisons are shaded grey.

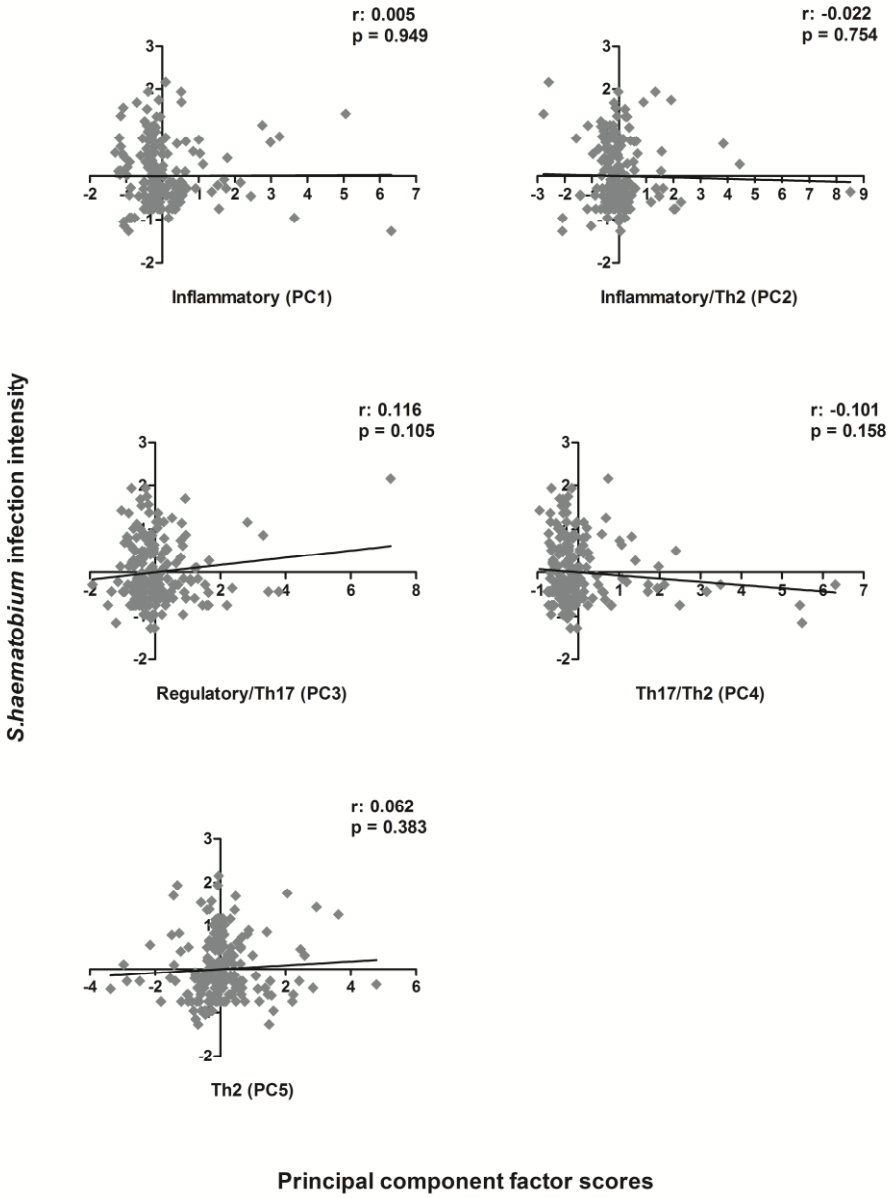


Figure 4.6. Pre-treatment infection intensity is not significantly correlated with GST-specific whole blood cytokine profiles. Residual variance in log10(x+1)-transformed infection intensity after accounting for sex, HIV status and age group is plotted against residual variance in participant factor scores for each PC extracted from pre-treatment GST-specific cytokine responses. The Pearson's r value and significance level of correlations are indicated for each plot and significant correlations are highlighted in bold and underlined. Black lines: best fit values from linear regression.

Factor *(degrees of freedom)*	***Infection intensity***		
	F	***p***	***Post-hoc comparisons***
Sex *(1, 190)*	12.173	**0.001**	**males > females**
HIV status *(1, 190)*	0.842	0.360	
Age group *(2, 190)*	10.258	**<0.001**	**11-12 > 5-10 years** **11-12 > 13+ years**
GST-specific cytokine profiles:			
PC1 (Inflammatory) *(1, 190)*	0.004	0.950	
PC1*Age group *(2, 190)*	0.995	0.372	
PC2 (Inflammatory/Th2) *(1, 190)*	0.095	0.758	
PC2*Age group *(2, 190)*	0.037	0.964	
PC3 (Regulatory/Th17) *(1, 190)*	2.607	0.108	
PC3*Age group *(2, 190)*	1.317	0.270	
PC4 (Th2/Th17) *(1, 190)*	1.970	0.162	
PC4*Age group *(2, 190)*	1.207	0.301	
PC5 (Th2) *(1, 190)*	0.748	0.388	
PC5*Age group *(2, 190)*	1.060	0.349	

Table 4.7. Variation in infection intensity is not associated with GST-specific cytokine profiles. Results of ANOVA of log10(x+1)-transformed mean *S. haematobium* egg counts/10ml compared according to factor scores of each cytokine profile (PC) after accounting for variation due to sex, HIV status and age group. The effects of each PC was analysed in separate ANOVA. Significant results ($p<0.05$) are highlighted in bold. None of the relationships identified were significant after Bonferroni correction for multiple comparisons.

4.5 Discussion

Schistosome infection intensity and the immune responses to parasite antigens are inter-dependent and both are influenced by age-related factors. Physiological changes with age. including 'maturation' of the immune system during early life (Grogan *et al.* 1996b), immunological effects of pubertal hormones (Fulford *et al.* 1998), immunosensescence in adulthood (Comin *et al.* 2008) and variation in water contact behaviour (Chandiwana and Woolhouse 1991; Chandiwana *et al.* 1991), appear to influence immune responses to schistosomes. However, in light of studies demonstrating that age-related exposure to schistosome infection rather than age alone may promote the development of parasite-specific immune responses (Mutapi *et al.* 1997), I sought to investigate whether parasite-specific cytokine responses could be related to epidemiological patterns of *S. haematobium* infection in Magaya community.

Consistent with the findings of most studies of human schistosomiasis, *S. haematobium* infection intensity followed a typical convex pattern with age. The study cohort was therefore sub-divided into 3 age groups that reflected this age-infection pattern (full details given in chapter 2.3.7). For life-long permanent residents of the study area (where *S. haematobium* transmission is stable and endemic) these age groups were indicators of both duration of exposure to schistosome infection (i.e. the youngest age group had the shortest and the oldest age group has the longest exposure history) and current infection levels (i.e. 11-12 year olds bore the highest mean infection intensity). From this cross-sectional distribution of infection by age it can be assumed that the 13+ age group is made up of individuals who have previously harboured high intensity infections that have now declined due to the development of resistance. In contrast, the 5-10 year olds are yet to reach peak infection intensity and are likely to acquire increasing numbers of parasites. Whilst this pattern is evident at a population level the range of infection intensities within each age group, particularly 11-12 year olds (0-692 eggs/10ml urine), suggests that there is also considerable variation in the age at which individuals reach their peak level of infection.

Since the immune environment during natural infection comprises cytokine responses elicited by numerous different antigens and secreted by a range of cell types whole blood cytokine responses to adult worm and egg antigens were grouped by factor analysis. Factor analysis has been successfully used to study immune responses to human helminth infections

previously, but these studies have focused on a limited repertoire of cytokines (Turner *et al.* 2003; Jackson *et al.* 2004b; Mutapi *et al.* 2007b), single or non-parasite antigens (Turner *et al.* 2003; Mutapi *et al.* 2007b) or systemic rather than parasite-specific responses (Milner *et al.* 2010). Thus, the current study provides an extension to this powerful approach to analysing immunology data. Analysis identified clear groupings of cytokines associated with innate inflammatory, Th1, Th2, Th17 and regulatory-type responses to antigens of both *S. haematobium* life-cycle stages. Furthermore, most of the cytokine profiles identified comprised cytokines associated with more than one of these phenotypes, consistent with growing evidence that schistosomes elicit a mixed cellular effector phenotype that cannot be adequately characterised by the Th1/Th2 paradigm alone (reviewed by (Allen and Maizels 1997; Diaz and Allen 2007)). In particular, positive loadings of Th17-type cytokines with inflammatory (PC1), regulatory (PC2) and Th2 (PC3)-type responses, indicated that cytokines associated with different cellular phenotypes are co-incident. IL-4 and IL-13, which are typically associated with Th2 responses, also had opposite loadings onto PC4, supporting observations in previous studies that Th2-type cytokines are not always co-expressed and may temporally dissociate during the course of infection (Ghaffar *et al.* 1997; Scott *et al.* 2000). The advantage of factor analysis in this context is that it identifies the cytokine patterns that account for the majority of variation in cytokine responses within the population, allowing this complexity to be explored as a smaller number of informative variables (Sokal and Rohlf 1995a). A mixed profile of pro-inflammatory cytokines (PC1) accounted for the greatest proportion of variation between participant cytokine responses to SEA and WWH, which reflects the high levels of these cytokines produced *in vitro* (identified in chapter 3). However, these responses were not significantly influenced by host sex, HIV status or age group and did not account for significant variation in *S. haematobium* infection levels. Thus heterogeneity in PC1 may reflect innate cellular responses to 'foreign' (or 'non-self') epitopes present in the WWH and SEA milieu (and potentially shared with CAP (Curwen *et al.* 2004)) rather than a cellular memory response conditioned by parasite exposure.

SEA and WWH-specific regulatory/Th17 (PC2) and Th17 (PC6)-type cytokine responses were significantly influenced by host age group and accounted for significant variation in *S. haematobium* infection intensity. PC2 responses (reflecting WWH and SEA-specific IL-10 and IL-21) were highest in the youngest age group (5-10 years) and PC6 responses (WWH and SEA-specific IL-17A) were higher in 5-10 and 11-12 year olds than in 13+ year olds.

The main distinction between the youngest and oldest age groups, which do not differ in their mean infection intensities or water contact behaviours, is their history of exposure to infection and age-dependent physiological changes which may affect their capacity to respond to *S. haematobium* antigens. Thus, it is possible that the lower levels of PC2 cytokine responses in 13+ year olds relative to the age groups where infection levels are increasing (5-10 years) or peaking (11-12 years) may contribute to their development of resistance to *S. haematobium*. The significant effect of the interaction between age group and infection intensity also indicates that the differences between PC2 responses according to age group were dependent on current levels of infection.

When the reciprocal influence of variation in cytokine profiles on *S. haematobium* egg counts was investigated, infection intensity was found to be positively correlated with PC2 responses after accounting for variation due to host sex, HIV status and age group. This pattern suggests that PC2 responses to adult worm and egg antigens may be induced by infection leading to high levels in heavily infected individuals but reflect an immune environment that is ineffective at clearing patent infection and/or limiting acquisition of new parasites. Alternatively, these responses may be important regulators of morbidity during heavy infections, but also limit parasite-specific effector responses (refer to recent reviews (Jackson *et al.* 2009; Maizels *et al.* 2009)). For example, IL-10-mediated suppression of effector cytokines has also been interpreted as mechanism for regulating egg-induced immunopathology in humans (Grogan *et al.* 1998b) and murine infection models (Hesse *et al.* 2004). Furthermore previous work has shown that a decline in mean IL-10 and reciprocal rise in mean IL-5 concentrations with age coincides with reduction in mean infection intensity in a population endemically-exposed to *S. haematobium* (Mutapi *et al.* 2007b).

In contrast to PC2 cytokine responses, infection intensity was negatively correlated with Th17-type (PC6) responses, suggesting that they may promote resistance to infection and/or be suppressed during infection. These findings agree with the only previous study to investigate IL-17 responses in an *S. haematobium*-exposed population, which found that IL-17A was more frequently detected in the plasma of un-infected individuals than their infected counterparts (Milner *et al.* 2010). However, as has been previously observed in plasma samples (Milner *et al.* 2010), only a small proportion of participants in the current study produced detectable levels of IL-17A (SEA n = 37/198, WWH n = 28/198, GST n = 39/198 produced IL-17A at concentrations above those present in un-stimulated cultures).

Furthermore, those participants who produced detectable levels of *S. haematobium*-specific IL-17A did so at low levels (mean SEA-specific IL-17A: 0.0127ng/ml (n = 37), mean WWH-specific IL-17A: 0.103ng/ml (n = 28), mean GST-specific IL-17A: 0.009ng/ml (n = 39)). Therefore, whilst the relationship between infection intensity and IL-17A identified in the current study may be of importance, further work will be required to increase the sensitivity of ELISA-based IL-17A detection in peripheral samples and thus increase the statistical power of future studies to investigate IL-17A responses in human schistosomiasis.

At first the opposite relationships with infection intensity of the Th17-associated cytokine profiles (i.e. regulatory/Th17 (PC2) and Th17 (PC6)) appear contradictory, however it is important to note that whilst both PCs were defined according to their association with the Th17 lineage, they are loaded with adult worm and egg-specific IL-21 (PC2) and IL-17A (PC6) respectively. Despite being described as a Th17 cytokine in recent human studies (chapter 2.3.5.5), the phenotype of IL-21-secreting cells is unclear and its functional role may differ between the human and murine immune systems (Wurster *et al.* 2002; Hoeve *et al.* 2006; Pesce *et al.* 2006). Different effects of IL-17A and IL-21 on *S. haematobium* infection levels may result from effector functions that are yet to be characterised in humans (e.g. induction of alternatively activated macrophages (Pesce *et al.* 2006)). It is also possible that co-variance between IL-10, a well characterised immunoregulatory cytokine, and IL-21 is due to their shared ability to suppress other cytokine responses (Grogan *et al.* 1998a; Wurster *et al.* 2002). However, the lack of previous studies characterising IL-17 and IL-21 responses relative to epidemiological patterns of helminth infection and their poorly defined mechanistic roles in human immunology mean that further studies are required to test these hypotheses.

In addition to the linear correlation between infection intensity and PC2 cytokine responses, infection intensity was also significantly affected by the interaction between PC2 responses and age group. This effect was due to a change from a significant positive correlation in 5-10 year olds to no significant correlation in 11-12 and 13+ year olds. A similar age-related change in correlation has been observed between helminth infection intensity and *S. haematobium*-specific IgG3 responses (Mutapi *et al.* 2006), the proportion of T regulatory (Treg) to T effector (Teff) cells in an *S. haematobium*-exposed cohort (Nausch *et al.* 2011), *Trichuris trichuria*-specific IL-10 (Turner *et al.* 2003) and *Necator americanus*-specific IgG (Quinnell *et al.* 1995). This changing relationship with age has been interpreted as an

indicator of immune-mediated resistance to infection developing with age and parasite exposure (Quinnell *et al.* 1995; Quinnell *et al.* 2004a; Mutapi *et al.* 2006). Mathematical models of infection predict that parasite-specific protective immune responses are initially positively correlated with infection intensity as they are stimulated by parasite antigens, but this correlation changes with increasing age as they exert their anti-parasite effects (Woolhouse 1994). Given the putative role of IL-10 in suppressing schistosome-specific effector immune responses, it seems unlikely that regulatory/Th17-type responses directly mediate anti-parasite immunity. It seems more likely that PC2 responses are up-regulated during infection in young children and subsequently decline due to cumulative antigen exposure, allowing protective effector responses to be up-regulated in later life. For example, the results presented here suggest that parasite-specific IL-17 responses are negatively correlated with infection intensity across all age groups, but the effector function of this response may be limited in young children with high levels of PC2 cytokines. The latter would correspond with previous studies proposing that the balance between immune effector responses and immunoregulatory responses may be a more important determinant of both anti-parasite and anti-pathology immunity to schistosomes than individual effectors (Corrêa-Oliveira *et al.* 1998; Hesse *et al.* 2004; Mutapi *et al.* 2007b; Nausch *et al.* 2011). Furthermore and in contrast to the suggestion that young children exhibit stereotypical immune responses to schistosome antigens as a result of their 'immature' immune response (Grogan *et al.* 1996b), regulatory/Th17-type cytokine responses were only higher than in other age groups in 5-10 year olds with heavy infections (50 eggs/10ml urine or more). Thus, these observations are consistent with the long-held hypothesis that natural immune-mediated resistance to schistosome infection is dependent on cumulative exposure to infection (chapter 1.2.4).

Cytokine profiles elicited by GST differed from those elicited by WWH and SEA, potentially due to the absence of other immunogenic antigens present in the crude WWH and SEA preparations. However, similar to SEA and WWH, the cytokine groupings identified in GST-stimulated cultures suggested a mixed profile of whole blood cytokine responses. Furthermore, analysis extracted both an inflammatory (GST PC1) component, reflecting TNFα, IL-6, IFNγ, IL-12p70 and IL-23 responses, and regulatory/Th17 (GST PC3)-type component , reflecting IL-10 and IL-21 responses, suggesting similarities in the cytokine profiles elicited by stimulation with GST, SEA and WWH. The parallels between the principal components identified for GST and SEA and WWH-stimulated cultures may be

due to the abundance and immunogenicity of GST, which is a significant component of both WWH and SEA (Curwen *et al.* 2004; Mutapi *et al.* 2005). Both GST-specific PC1 and PC3 had similar patterns with age group to those of their SEA and WWH-specific counterparts (PC1 and PC2 respectively). GST PC1 cytokine responses were significantly higher in 11-12 year olds, who bore the highest mean infection intensity, than in 5-10 or 13+ year olds, suggesting that levels of inflammatory cytokines produced *in vitro* are closely related to current infection levels (this pattern was the same, though not statistically significant, for SEA and WWH-specific inflammatory cytokines). GST PC3 cytokine responses were highest in 5-10 year olds, but this difference was only significant when compared to 11-12 year olds. None of the GST-specific cytokine profiles identified were significantly affected by the interaction between age group and infection intensity or correlated with *S. haematobium* egg counts. Thus, despite the similarities with SEA and WWH-specific responses, the GST-specific cytokine profiles were not indicators of resistance to infection within the cohort. The increase in sero-reactivity to GST isoforms within crude *S. haematobium* WWH after praziquantel treatment (Mutapi *et al.* 2005), suggests that treatment increases exposure to parasite antigens and the effect of treatment on GST-specific cytokine responses is investigated further in chapters 5 and 6. An alternative hypothesis is that induction of GST-specific antibodies rather than the type of cytokine profile that it elicits *in vitro* are better indicators of protective immunity (Lane *et al.* 1998; Capron *et al.* 2001). It is possible that, although GST vaccination can elicit protective immunity in primates (Boulanger *et al.* 1995; Boulanger *et al.* 1999), the amount of GST to which human hosts are exposed during natural infections is insufficient to generate cytokine responses associated with anti-parasite immunity. Alternatively, GST-specific antibody responses may provide a better indicator than cytokine responses of the change in GST-specific immune responses with age and exposure to infection, as has been proposed by others (Mutapi *et al.* 2008). Thus, although the results presented here suggest that whole blood cytokine responses to GST *in vitro* are not indicative of naturally-acquired resistance to high worm burdens, they do not preclude the possibility that GST administered as a vaccine could artificially induce protective immunity (Dupre *et al.* 1999).

A final consideration is that the GST used (purified from a Senegalese strain of *S. haematobium*) and the SEA and WWH (prepared from Egyptian parasites) may elicit different cytokine profiles than those of the Zimbabwean schistosomes to which Magaya community are exposed. There is known to be antigenic variation between schistosome

species (Trottein *et al.* 1992a) and it has also been proposed to occur between intra-specific strains by theoretical models of infection (Galvani 2005). However, clear patterns of cytokine responses to these antigens with age and infection intensity and the known cross-reactivity of antigens derived from the same Egyptian parasites with serum antibodies from individuals endemically exposed to Zimbabwean *S. haematobium* (Mutapi *et al.* 2003; Mutapi *et al.* 2008; Mutapi *et al.* 2011a), suggest that immune responses are raised against antigens shared across the different parasite strains.

4.6 Conclusions

The results of the current study are consistent with the initial hypotheses that parasite-specific cytokine responses vary with age and infection intensity and may also have reciprocal effects on infection levels. In particular, the 3-way association identified between age group, infection intensity and the combination of IL-10 and IL-21 (regulatory/Th17) responses to *S. haematobium* adult worm and egg antigens provides evidence that whole blood cytokine responses elicited *in vitro* are indicators of both exposure history and the development of immune-mediated resistance to infection. The change in association between infection intensity and regulatory/Th17-type cytokines with age suggests that 5-10 year olds, in whom infection is increasing, have a distinct cytokine response to infection than age groups experiencing peaking and declining levels of infection. The putative function of IL-10 suggests that it may serve anti-pathology functions and/or limit protective effector responses in young children with high intensity infections and decline as resistance develops with age. Conversely, adult worm and egg-specific IL-17A may contribute to immune-mediated resistance to schistosome infection independent of variation due to age group alone.

This study is the first to identify how *S. haematobium*-specific Th17-associated cytokines may contribute to epidemiological patterns of infection. The association of IL-17A, IL-21 and IL-23 with distinct combinations of innate inflammatory, Th1 and Th2-type responses suggests that these cytokines may have a range of effector functions in human schistosomiasis. Further study of these cytokines and their cellular sources is warranted to test the relevance of these findings to human disease.

Chapter 5

The effect of anti-helminthic treatment on whole blood cytokine responses to *Schistosoma haematobium* antigens

5.1 Introduction

Praziquantel treatment effectively clears schistosome infection, but also alters the host immune response (chapter 1.8.3). In this chapter I will explore whether *S. haematobium* cercariae, adult worm, egg and glutathionine-S-transferase (GST)-specific whole blood cytokine responses are altered 6 weeks after treatment. By this time-point praziquantel is fully metabolised (Na-Bangchang *et al.* 2006; Njomo *et al.* 2010) and the reduction in urine egg counts is maximal (Tchuem Tchuente *et al.* 2004), but newly acquired infections are yet to reach patency (Loker 1983), allowing cytokine responses to be investigated in the absence of chronic infection.

I first sought to extend the observations of previous studies which have shown that cytokine responses to schistosome antigens differ after treatment relative to pre-treatment levels (Roberts *et al.* 1987; van den Biggelaar *et al.* 2002; Fitzsimmons *et al.* 2004; Joseph *et al.* 2004b; Reimert *et al.* 2006), by assaying a broader range of schistosome antigen-specific cytokine responses. I then investigated whether, in addition to altering individual cytokine responses, treatment leads to a shift in the overall schistosome-specific cytokine profile of endemically-exposed individuals. The latter has been inferred from previous observations showing that IFNγ and IL-5 responses are inversely associated (Roberts *et al.* 1993), but has never been investigated in more than 2 cytokines. Importantly, a combined analysis of pre and post-treatment innate inflammatory, Th1, Th2 and Th17-type cytokine responses allows the major sources of variation in the host cytokine phenotype resulting from removal of schistosome infection to be identified.

In light of pre-treatment differences in the cytokine responses to cercariae, adult worm and egg-stage parasites (chapter 3), I have also addressed the hypothesis that the relative cytokine profiles elicited by these 3 life cycle stages differ 6 weeks after treatment. Different

effects of praziquantel treatment on cytokine responses to different schistosome life-cycle stages might result from variation in the efficacy of the drug against each stage and/or removal of stage-specific immunosuppression present during chronic infection. In particular, praziquantel is proposed to target mainly adult parasites, however there is evidence from both murine models (Xu *et al.* 1988; Flisser *et al.* 1989; Shaw 1990; Giboda and Smith 1994) and human studies (Mutapi *et al.* 1998b; Caldas *et al.* 2000; Guidi *et al.* 2010) that treatment may also alter immune responses to immature parasites.

5.2 Hypotheses

- Individual *S. haematobium*-specific cytokine responses are altered 6 weeks after praziquantel treatment

- Praziquantel treatment causes a shift in the *S. haematobium*-specific cytokine profile

- *S. haematobium*-specific cytokine profiles 6 weeks after treatment differ between the different parasite life-cycle stages (cercariae, adult worms and eggs)

5.3 Materials and Methods

5.3.1 Study participants

Of the participants recruited from Magaya community before treatment, 94 provided a follow-up sample for whole blood culture 6 weeks after treatment and met the inclusion criteria for the current study: 1) tested negative for *S. mansoni*, soil-transmitted helminth (STH), malaria and HIV both before and after treatment, 2) provided a minimum of 2 stool and 2 urine samples for microscopic analysis of helminth egg counts both before and after treatment, 3) reported no prior treatment for schistosomiasis and 4) were long-term, permanent residents of the area. In addition all participants had received a single standard dose 40mg/kg body weight praziquantel (for schistosome infection) and 400mg albendazole (for STH infection) irrespective of their infection status according to the recommendations of the World Health Organisation (WHO) (Montresor *et al.* 2002) and the ethical permissions obtained for the study (chapter 2.3.4). To exclude participants who harboured persistent infections, all participants included in this chapter were confirmed to be *S. haematobium* negative 6 weeks after treatment (i.e. successfully cleared of infection) via microscopic analysis of *S. haematobium* egg counts in filtered urine (chapter 2.3.5).

5.3.2 Immunological assays

Whole blood cultures were conducted in parallel with CAP, WWH, SEA and GST for each participant under the same conditions (10µg/ml crude antigen (CAP, WWH and SEA) or 2µg/ml GST at 37°C for 48 hours in OXOID Anaerobic™ pouches) both before and 6 weeks after treatment. Un-stimulated (culture media without antigen) whole blood cultures acted as negative controls for levels of spontaneous cytokine. Cultures stimulated with 10µg/ml Phytohaemagglutinin (PHA) acted as positive controls, confirming that cells were viable and capable of secreting cytokines when stimulated with antigen. For participants who did not provide sufficient amounts of blood for all antigen stimulations (e.g. due to participant discomfort during blood collection), cultures were prioritised as follows: SEA, WWH, GST, control cultures and CAP. For this reason, of the 94 individuals included in this chapter, CAP-specific cytokine responses were only assayed in 24 participants.

ELISA was used to quantify IFNγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-21 and IL-23p19 in culture supernatants relative to a pre-optimised standard curve of recombinant cytokine. A mean cytokine concentration (ng/ml) was obtained from duplicate ELISA wells for each antigen stimulation.

5.3.3 Statistical Analyses

To address the hypothesis that individual cytokine responses to *S. haematobium* antigens differ 6 weeks post-treatment relative to pre-treatment levels, all SEA, WWH and GST-specific (n = 94) and CAP-specific (n=24) cytokines were compared between the pre- and post-treatment cultures via repeated measures ANOVA. Square-root(x+1)-transformed cytokine responses were the dependent variables and time point (pre- and 6-weeks post-treatment) was included as a between-subject factor. The repeated measures analysis was chosen to account for re-sampling of the same individuals before and after treatment and comparison of the same cohort of participants before and after treatment meant that variation due to sex, age, pre-treatment *S. haematobium* infection status and intensity, genetic factors etc. were accounted for in the analysis. A separate repeated measures ANOVA of the non-specific cytokine concentrations present in un-stimulated control cultures was also conducted to investigate whether the systemic cytokine environment was affected by treatment. Fisher's least significant difference test was used for post-hoc pair-wise comparisons between pre and post-treatment cytokine responses in antigen-stimulated and un-stimulated cultures and the t-ratio (T) and associated p-value are reported for each test (Sokal and Rohlf 1995b). Exploratory analysis indicated that the square-root(x+1)-transformed cytokine data and residuals of ANOVA met parametric assumptions for both antigen-specific and un-stimulated responses.

To investigate whether the treatment-induced changes in each participant's combined cytokine response non-metric multidimensional scaling (NMS) was conducted for each antigen using PC-ORD software. This approach allowed post-treatment cytokine responses to be visualised relative to pre-treatment patterns via ordination plots. Full details of NMS procedure are given in chapter 2.5.4. Briefly, square-root(x+1)-transformed concentrations of all 13 cytokines were entered for all participants both before and after treatment and the dissimilarity (Sorensen distance) between each participant according to their combination of cytokine responses was calculated, ranked and plotted according to 2-dimensional axes by

ordination. The data was transformed in order to reduce the weight of outliers on the ordination solutions without affecting relative ranks of each participant (Rummel 1970). The axes reflected the cytokines responsible for the greatest variation between cytokine profiles. Starting coordinates for each ordination were selected using a random number generator and Solutions achieved with a final stress of less than 20 were considered to adequately represent interpretable patterns of cytokine responses (McCune and Grace 2002). The cytokine responses accounting for the greatest variation in participant cytokine profiles (i.e. correlated most strongly with the extracted axes) were identified by Pearson's 2-tailed correlation analysis

Cluster patterns of participant cytokine responses in NMS ordination plots provided a qualitative indicator of variation between participant cytokine responses. Differences between the mean dissimilarities of participant cytokine profiles before (i.e. the variation between all individuals' pre-treatment cytokine profiles) and 6 weeks after treatment were plotted for final NMS solutions and compared via multiple response permutation procedure (MRPP) in PC-ORD software. This analysis specifically tests the hypothesis that variation between pre- and post-treatment cytokine responses is greater than the variation in cytokine responses between individuals at either of these timepoints (McCune and Grace 2002). Homogeneity in cytokine profiles within pre and post-treatment groups was quantified via the chance corrected within-group agreement statistic (A), an estimate of 'effect-size' for the differences identified by MRPP. Given the size of our study cohort, an A <0.1 was considered as low and A> 0.3 was considered high (McCune and Grace 2002).

To investigate whether CAP, WWH and SEA elicit distinct cytokine profiles 6 weeks after treatment, the square-root(x+1) transformed post-treatment cytokine concentrations of each participant to all 3 antigens were first reduced into principal components (cytokine profiles) by factor analysis (Sokal and Rohlf 1995c). The effect of antigen (i.e. CAP, WWH and SEA) on each cytokine profile was then assessed via ANOVA. Only participants for whom CAP, WWH and SEA-stimulated cultures were conducted were included in the analysis (n = 24). Thus variance in the PCs due to host heterogeneity was accounted for by comparing the responses of the same 24 individuals to all antigens. Analysis was conducted in exactly the same way as that of pre-treatment CAP, WWH and SEA-specific cytokine responses in chapter 3 and exploratory analysis indicated that the regression factor scores met parametric assumptions.

The sequential Bonferroni correction was used to identify results that were significant in the context of the multiple statistical comparisons being made (Holm 1979; Rice 1989). Results significant after correction are reported alongside raw p-values for all analyses involving multiple comparisons.

5.4 Results

5.4.1 Differences between individual parasite-specific whole blood cytokine responses before and 6 weeks after treatment

Comparisons of mean cytokine levels elicited before and after treatment identified distinct dynamics according to antigen stimulation. Levels of all cytokine responses, with the exception of IL-5, to egg antigens were higher 6 weeks post-treatment relative to pre-treatment levels and this increase was statistically significant for innate inflammatory (TNFα, IL-6 and IL-8) and Th1-type (IFNγ, IL-2 and IL-12p70) cytokines and IL-23. GST-stimulation also elicited higher mean TNFα, IL-6, IL-8, IL-12p70 and IL-23 responses. Mean CAP-specific IL-2, IL-8 and IL-10, responses were higher, but IL-23 was significantly lower in post-treatment cultures. WWH-specific innate inflammatory cytokines (TNFα, IL-6 and IL-8) and IL-10 were lower post-treatment. For all antigen-stimulated cultures mean levels of IL-5 were lower, although this was not statistically significant for SEA-stimulated cultures. Mean IL-21 concentrations were also higher post-treatment, but only significantly so for CAP and WWH-stimulation. Mean IL-13 concentrations were significantly higher in response to all antigens, except WWH. These observations are consistent with cytokine responses to the different schistosome antigens being differentially affected by treatment. The changes in mean innate inflammatory, Th1, Th2 and Th17-type cytokine concentrations following treatment are summarised in Table 5.1 and plotted by antigen in Figures 5.1, 5.2, 5.3 and 5.4 respectively. Results of repeated measures ANOVA of pre and post-treatment cytokine responses are given in Table 5.2 and significant differences from this analysis are indicated in Figures 5.1-5.4.

Treatment-related changes in cytokine secretion in un-stimulated culture were also observed, reflecting changes in the systemic cytokine environment. Levels of spontaneous IL-8, IL-2 and IL-5 were lower and IL-12p70, IL-13 and IL-21 responses were higher post treatment relative to pre-treatment levels. Post-treatment changes in cytokine levels present in un-stimulated cultures are summarised in Table 5.1 and systemic pre and post-treatment cytokine responses are plotted in Figure 5.5. Repeated measures ANOVA of cytokine responses in un-stimulated cultures are summarised in Table 5.2.

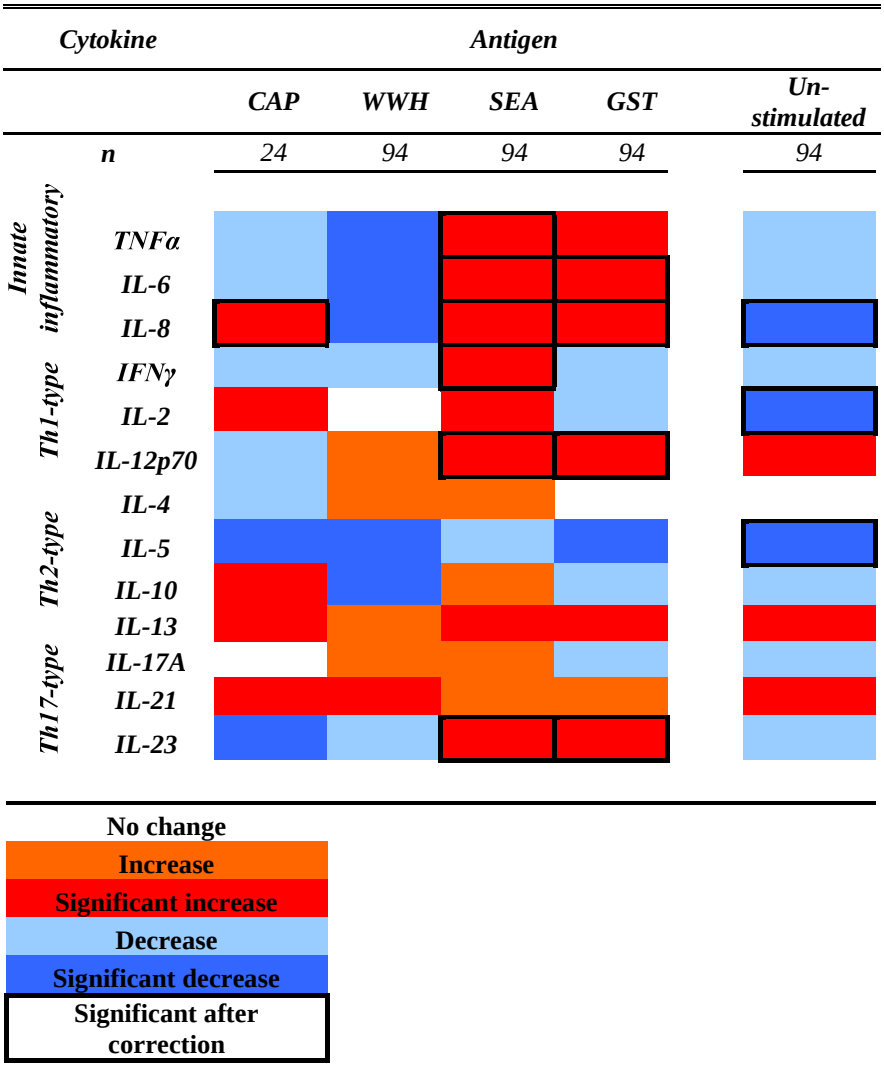


Table 5.1. Change in mean cytokine concentrations present in antigen-stimulated and un-stimulated cultures 6 weeks post-treatment. Colours indicate the direction of change in cytokine concentrations for the cohort after treatment relative to pre-treatment levels (red/orange – increase, blue – decrease, white – no change). Significant results are highlighted by darker colours. Results significant after Bonferroni correction for multiple comparisons are indicated by black borders. Only 24 of the 94 participants provided sufficient volumes of whole blood for CAP cultures to be conducted.

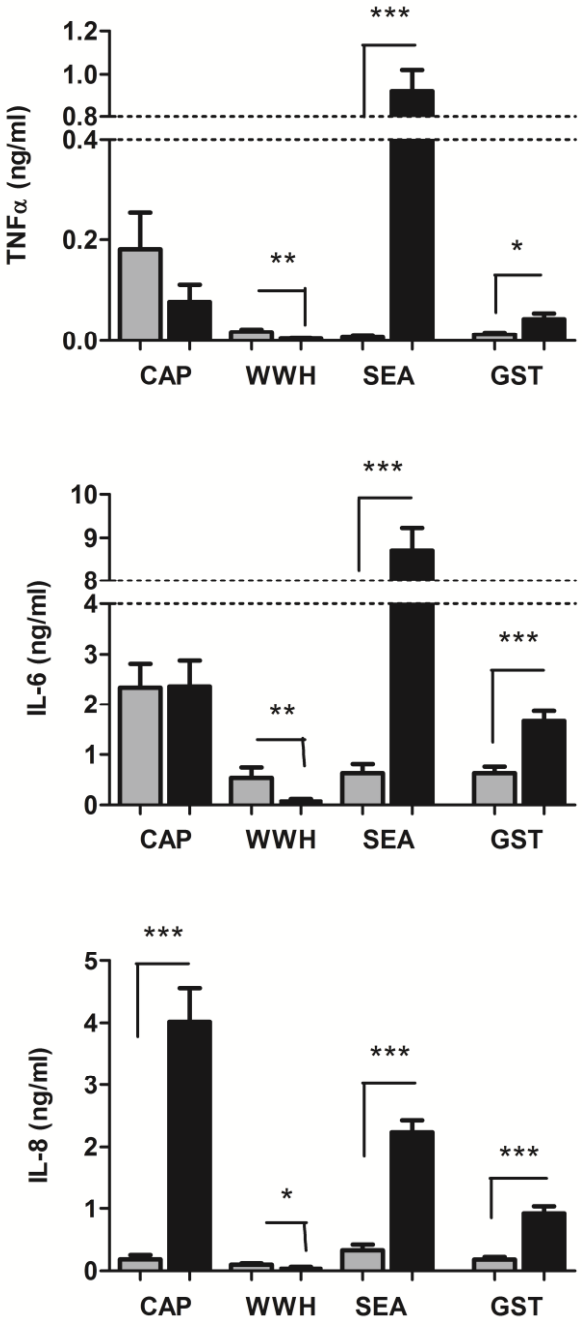


Figure 5.1. Innate inflammatory cytokine responses to cercariae, egg antigens and GST, but not adult worm antigens, increase 6 weeks after treatment. Bar charts represent mean un-transformed concentrations of antigen-specific cytokines (ng/ml) before (grey) and 6 weeks after (black) treatment. Error bars: standard error of the mean. Significant results of repeated measures ANOVA are indicated. *p<0.05, **p<0.01, ***p<0.001.

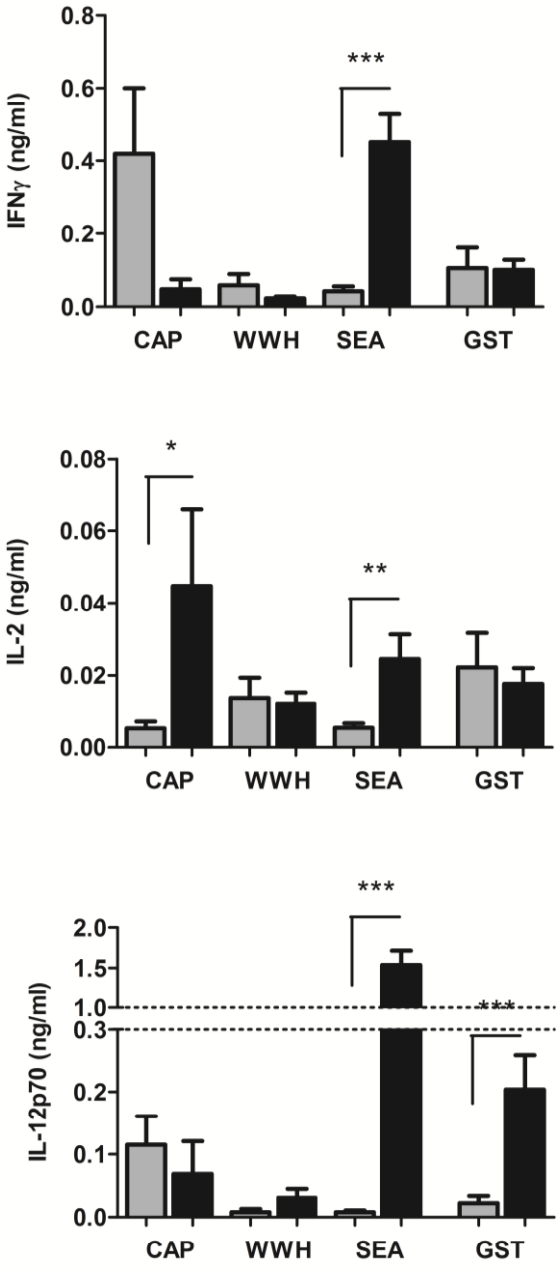


Figure 5.2. Th1-type cytokine responses to cercariae and egg antigens and GST, but not adult worm antigens, increase 6 weeks after treatment. Bar charts represent mean un-transformed concentrations of antigen-specific cytokines (ng/ml) before (grey) and 6 weeks after (black) treatment. Error bars: standard error of the mean. Significant results of repeated measures ANOVA are indicated. *p<0.05, **p<0.01, ***p<0.001.

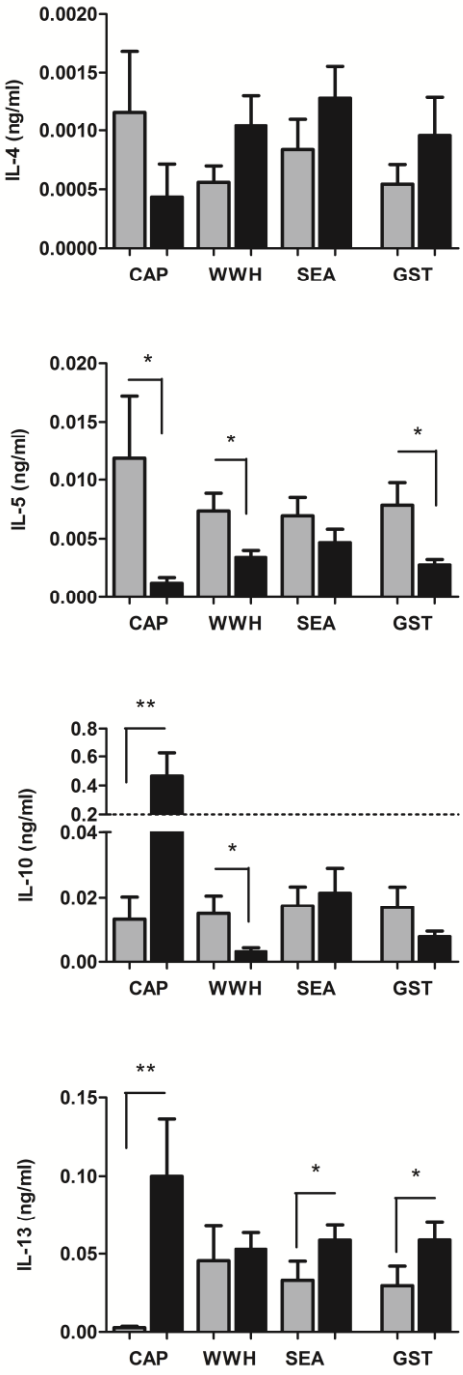


Figure 5.3. Th2-type cytokine responses differ 6 weeks after treatment relative to pre-treatment levels. IL-5 responses decrease and IL-13 responses increase. Changes in post-treatment IL-10 responses are antigen-dependent. Bar charts represent mean un-transformed concentrations of antigen-specific cytokines (ng/ml) before (grey) and 6 weeks after (black) treatment. Error bars: standard error of the mean. Significant results of repeated measures ANOVA are indicated. *p<0.05, **p<0.01, ***p<0.001.

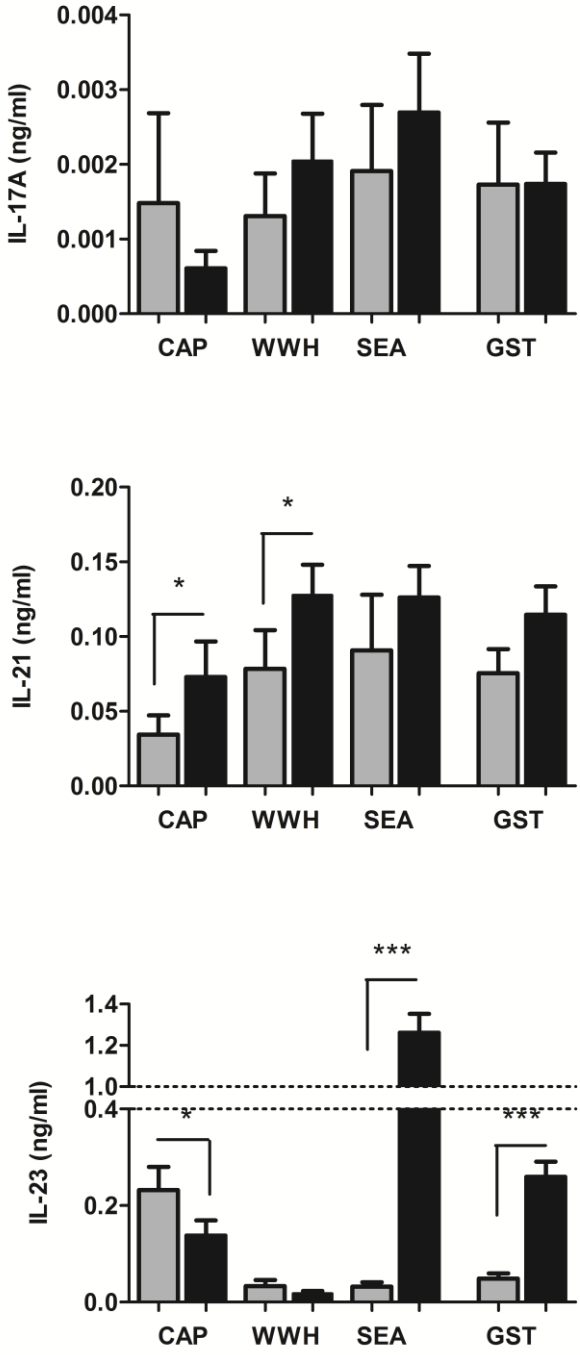


Figure 5.4. Th17-type cytokine (IL-21 and IL-23) concentrations **are higher 6 weeks after treatment than before treatment.** Bar charts represent mean un-transformed concentrations of antigen-specific cytokines (ng/ml) before (grey) and 6 weeks after (black) treatment. Error bars: standard error of the mean. Significant results of repeated measures ANOVA are indicated. *p<0.05, **p<0.01, ***p<0.001

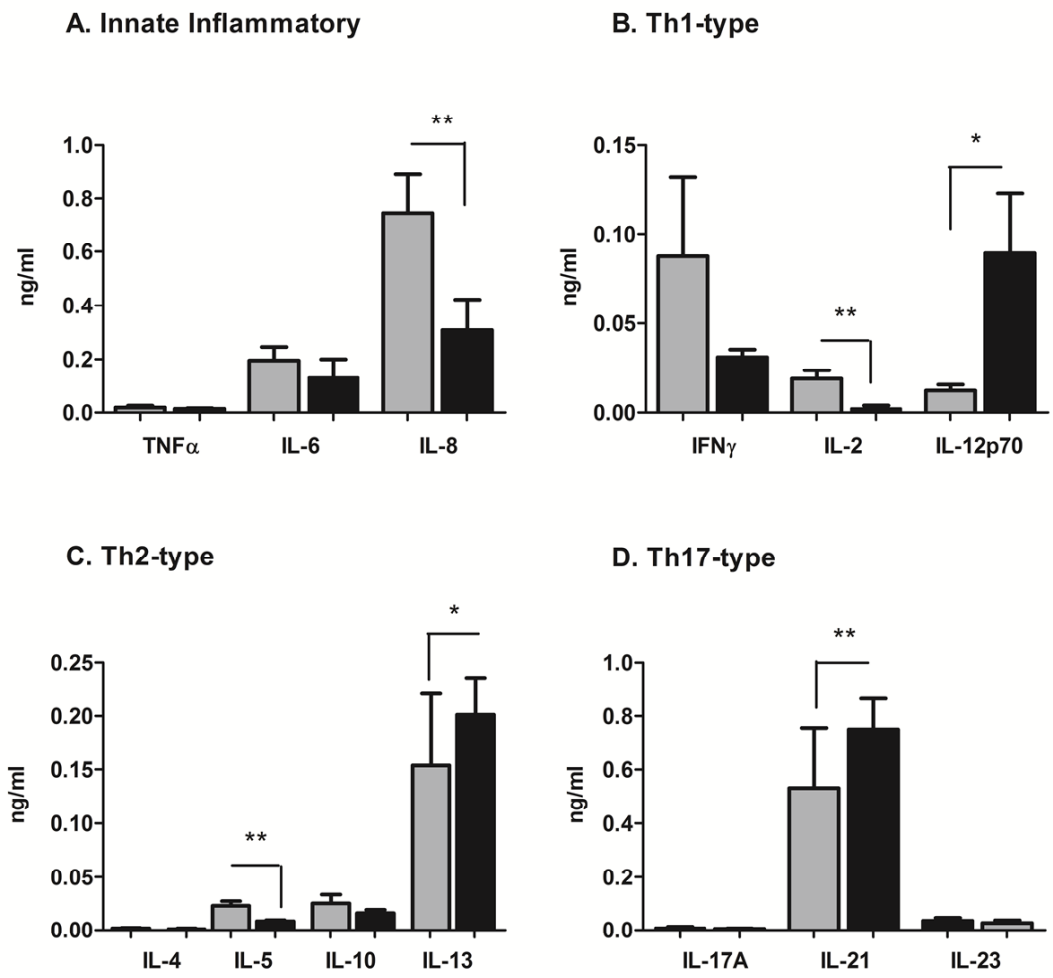


Figure 5.5. Cytokine concentrations in un-stimulated whole blood cultures differed 6 weeks after praziquantel treatment relative to pre-treatment levels. Bar charts show mean un-transformed concentrations of innate inflammatory (A), Th1 (B), Th2 (C) and Th17 (D)-type cytokines before (grey) and 6 weeks after (black) treatment present in cultures without antigen. Error bars: standard error of the mean. Significant results of repeated measures ANOVA are indicated. *p<0.05, **p<0.01, ***p<0.001

		Innate Inflammatory			*Th1-type*			*Th2-type*				*Th17-type*		
		TNFα	***IL-6***	***IL-8***	***IFNγ***	***IL-2***	***IL-12p70***	***IL-4***	***IL-5***	***IL-10***	***IL-13***	***IL-17A***	***IL-21***	***IL-23***
CAP	***F***	3.737	0.671	72.703	3.503	4.351	2.321	1.420	4.315	10.570	8.307	0.111	5.240	6.023
	p	0.066	0.421	**<0.001**	0.074	**0.048**	0.141	0.246	**0.049**	**0.004**	**0.008**	0.742	**0.032**	**0.022**
WWH	***F***	7.277	7.506	6.878	1.333	0.000	3.362	3.744	5.008	4.282	0.691	1.153	4.948	1.964
	p	**0.008**	**0.007**	**0.010**	0.251	0.993	0.070	0.056	**0.028**	**0.041**	0.408	0.286	**0.029**	0.164
SEA	***F***	97.746	286.579	124.277	33.380	8.326	119.479	1.895	1.219	0.169	4.295	0.563	2.175	260.315
	p	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.005**	**<0.001**	0.172	0.272	0.682	**0.041**	0.455	0.144	**<0.001**
GST	***F***	6.630	37.056	66.596	0.004	0.055	15.254	0.903	5.792	1.793	5.226	0.290	3.820	17.126
	p	**0.012**	**<0.001**	**<0.001**	0.951	0.816	**<0.001**	0.344	**0.018**	0.184	**0.025**	0.591	0.054	**<0.001**
Un-stimulated	***F***	1.401	1.980	10.839	1.707	11.038	5.719	0.211	10.966	1.106	4.750	0.791	7.122	1.421
	p	0.240	0.163	**0.001**	0.195	**0.001**	**0.019**	0.647	**0.001**	0.296	**0.032**	0.376	**0.009**	0.236

Table 5.2. Mean cytokine concentrations 6 weeks after treatment differ relative to pre-treatment levels. Table gives F statistic and p-value from repeated measures ANOVA comparison of the same participants pre and post-treatment (WWH, SEA, GST and un-stimulated, n = 94 and CAP, n = 24). Degrees of freedom, error degrees of freedom: 1, 93 (WWH, SEA, GST & Un-stimulated) and 1, 23 (CAP). Significant results ($p<0.05$) are highlighted in bold and those significant after Bonferroni adjustment for multiple comparisons are shaded grey. Direction of the change in cytokine concentrations is plotted in Figure 5.1-5.6 and summarised in Table 5.1.

5.4.3 Relative patterns of whole blood cytokine profiles before and after treatment

Having identified differences between the mean concentrations of individual cytokines following treatment, the implication of these changes for the whole blood cytokine environment (i.e. combination of cytokine responses) were investigated in all individuals via NMS. Ordination plots for each cytokine are shown in Figure 5.6 and the strength and direction of cytokine correlations with each ordination axis are summarised in Table 5.3.

Pre and post-treatment responses to CAP varied according to 2 axes (Figure 5.6.1) which negatively correlated with IL-8 (Axis 1) and negatively correlated with a combination of TNFα and IL-6 (Axis 2). Distinct pre and post-treatment clusters along Axis 1 reflect the increase in cercariae-specific IL-8 6 weeks after treatment (Figure 5.1). Thus, of the changes in individual cytokine responses to cercariae antigens, the majority of variation between individuals post-treatment is attributable to changes in their IL-8 response. MRPP comparison of responses before and after treatment confirmed that participant cytokine profiles significantly differed following treatment (T statistic: -19.40, $p<0.001$, A: 0.205).

Variation between pre and post-treatment cytokine responses to WWH (Figure 5.6.1) was mainly due to pre-treatment IL-6 responses (Axis 1). None of the pre or post-treatment cytokine responses were strongly associated with Axis 2 and thus, WWH-specific cytokine responses were best characterised by a 1-dimensional ordination solution (Table 5.3). Although MRPP indicated that WWH-specific cytokine profiles changed following treatment (T statistic: -11.89, $p<0.001$, A: 0.026), the low value of the chance-corrected within-group agreement (A) reflects that pre and post-treatment differences are small and do not occur in all participants.

SEA-specific cytokine responses exhibited a clear shift after treatment along 2 axes (Figure 5.6.2); TNFα, IL-6, IL-8, IL-12p70 and IL-23 (Axis 1) and TNFα, IL-6, IL-12p70 and IL-23 (Axis 2). 6 weeks after treatment the inflammatory cytokine responses on both axes were higher than those before treatment and the distinct clustering and low overlap of cytokine profiles according to treatment status (pre and post-treatment) indicates that this shift was evident in all participants. The loading of IL-8 onto axis 1, but not axis 2 indicates that

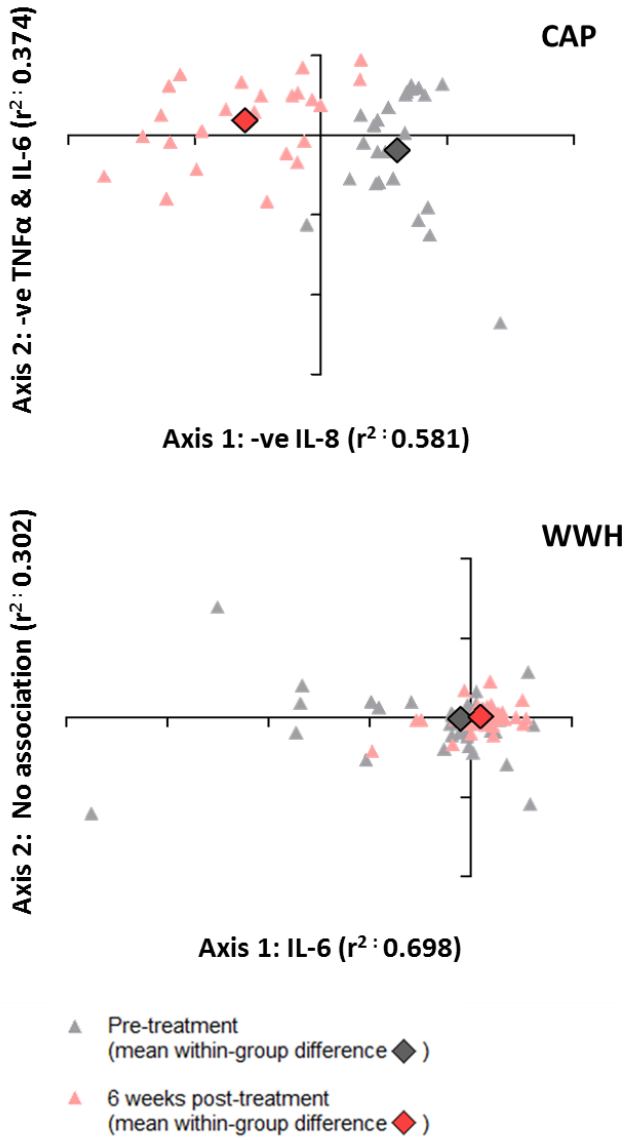


Figure 5.6.1 Cytokine profiles elicited by *S. haematobium* cercariae differ 6 weeks post-treatment relative to pre-treatment responses, but this change is less marked in adult worm-specific cytokine responses. NMS ordination plots generated from Sorensen Bray-Curtis distances between participant cytokine responses to CAP (top) and WWH (bottom) plotted according to 2-dimensional axes. The proportion of variance in participant cytokine responses attributable to each axis (Pearson's r^2) is shown. Axis 2 of the WWH-specific cytokine ordination plot was not strongly associated with any of the pre or post-treatment cytokine responses assayed. Axes are labelled according to the cytokines with which they are most strongly correlated (Table 5.2). Mean within-group differences reflect the total variation between participant cytokine responses before (grey) and after (red) treatment.

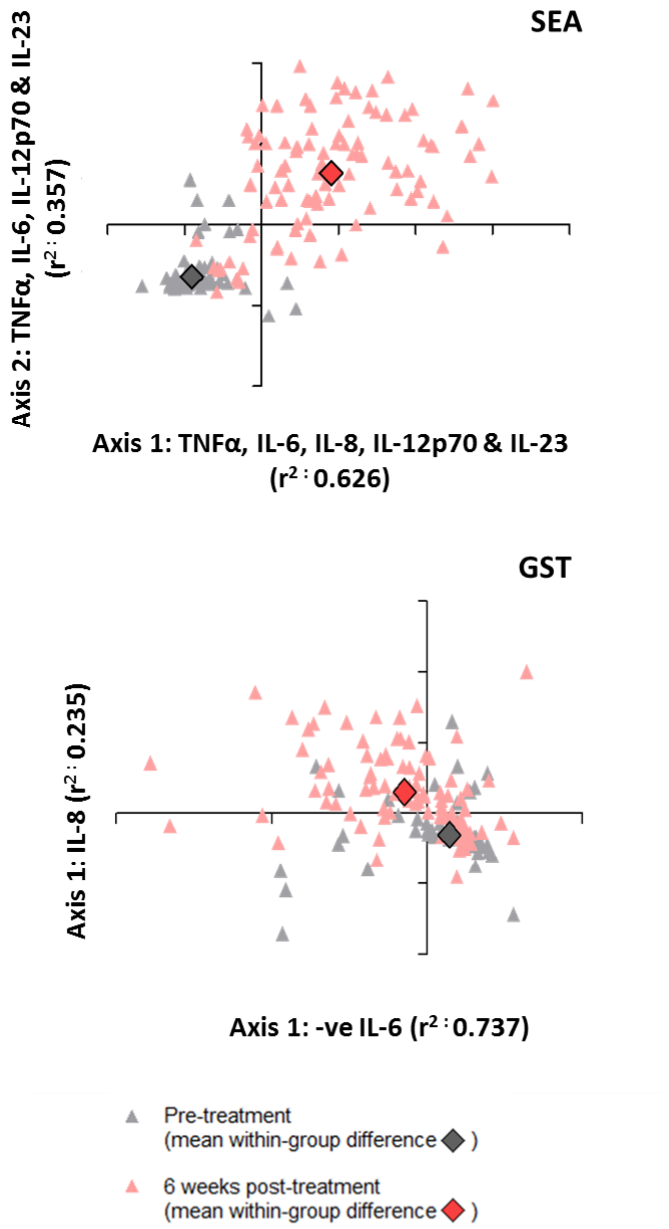


Figure 5.6.2 Cytokine profiles elicited by *S. haematobium* egg antigens and GST differ 6 weeks post-treatment relative to pre-treatment responses. NMS ordination plots generated from Sorensen Bray-Curtis distances between participant cytokine responses to SEA (top) and GST (bottom) plotted according to 2-dimensional axes. The proportion of variance in participant cytokine responses attributable to each axis (Pearson's r^2) is shown. Axes are labelled according to the cytokines with which they are most strongly correlated (Table 5.2). Mean within-group differences reflect the total variation between participant cytokine responses before (grey) and after (red) treatment.

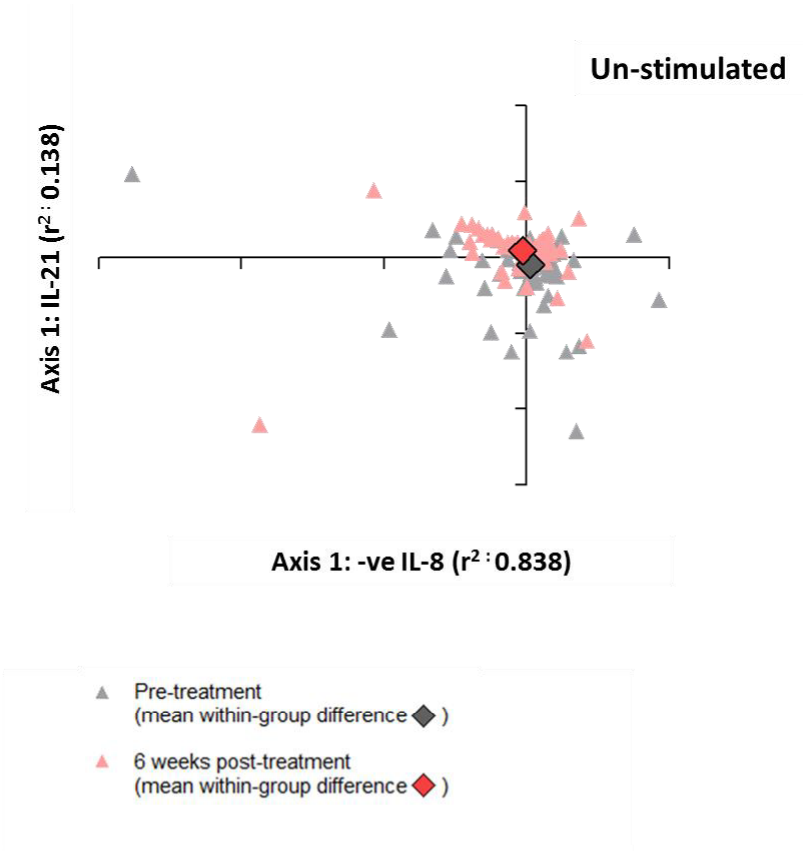


Figure 5.6.3 Spontaneous cytokine production by peripheral blood cells differs 6 weeks post-treatment relative to pre-treatment responses. NMS ordination plot generated from Sorensen Bray-Curtis distances between participant cytokine responses in un-stimulated whole blood cultures plotted according to 2-dimensional axes. The proportion of variance in participant cytokine responses attributable to each axis (Pearson's r^2) is shown. Axes are labelled according to the cytokines with which they are most strongly correlated (Table 5.2). Mean within-group differences reflect the total variation between participant cytokine responses before (grey) and after (red) treatment.

	Cytokine	Antigen																	
		CAP				WWH		SEA				GST				Un-stimulated			
		Axis 1		Axis 2		Axis 1		Axis 1		Axis 2		Axis 1		Axis 2		Axis 1		Axis 2	
		r	r^2	*r*	r^2	*r*	r^2	*r*	r^2	*r*	r^2	*r*	r^2	*r*	r^2	*r*	r^2	*r*	r^2
Innate inflammatory	***TNFα***	0.3	0.1	**-0.8**	**0.6**	0.4	0.2	**0.7**	**0.5**	**0.8**	**0.6**	-0.6	0.3	0.2	0.1	-0.1	0.0	0.3	0.1
	IL-6	0.0	0.0	**-0.9**	**0.9**	**0.7**	**0.5**	**0.8**	**0.6**	**1.0**	**0.9**	**-0.9**	**0.9**	0.5	0.2	-0.6	0.4	0.6	0.3
	IL-8	**-0.9**	**0.9**	0.2	0.0	0.0	0.0	**0.9**	**0.8**	0.5	0.2	-0.3	0.1	**0.9**	**0.8**	**-0.8**	**0.6**	0.4	0.2
Th1-type	***IFNγ***	0.3	0.1	-0.6	0.3	0.4	0.2	0.4	0.2	0.6	0.4	-0.5	0.2	-0.2	0.0	-0.1	0.0	-0.2	0.0
	IL-2	-0.2	0.0	0.2	0.0	-0.1	0.0	0.2	0.1	0.1	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
	IL-12p70	0.3	0.1	-0.7	0.4	0.2	0.0	**0.7**	**0.5**	**0.8**	**0.6**	-0.6	0.4	0.1	0.0	0.0	0.0	-0.1	0.0
Th2-type	***IL-4***	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	-0.1	0.0	0.0	0.0	0.0	0.0
	IL-5	0.2	0.1	-0.1	0.0	-0.1	0.0	0.0	0.0	-0.1	0.0	0.1	0.0	-0.1	0.0	0.0	0.0	0.0	0.0
	IL-10	-0.6	0.3	-0.1	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.0	-0.1	0.0	0.0	0.0	0.3	0.1
	IL-13	-0.4	0.2	0.1	0.0	-0.1	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.4	0.1	0.5	0.3
Th17-type	***IL-17A***	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.0
	IL-21	-0.2	0.0	0.1	0.0	0.0	0.0	0.2	0.0	0.1	0.0	-0.1	0.0	0.1	0.0	0.5	0.2	**0.8**	**0.6**
	IL-23	0.2	0.0	-0.4	0.2	0.2	0.0	**0.8**	**0.7**	**0.9**	**0.8**	-0.5	0.3	0.3	0.1	-0.6	0.3	0.3	0.1
	Dimensions	2				1		2				2				2			

Table 5.3. Sources of variation between schistosome-specific cytokine profiles before and 6 weeks after praziquantel treatment. Axes were identified by NMS of cytokine responses in CAP, WWH, SEA and GST and un-stimulated whole blood cultures. The Pearson's correlation coefficient (r), indicating the direction of the association of each cytokine with NMS axes, and coefficient of determination (r^2), indicating the proportion of variance along an axis attributable to each cytokine, is given. Cytokines with an $r^2 \geq 0.5$ are highlighted in bold and shaded grey. The dimensions of the final solution are shown below each column. Corresponding ordination plots are shown in Figure 5.6.

participants differ in their IL-8 responses, despite the uniform up-regulation of inflammatory cytokines after treatment. MRPP confirmed that egg-specific pre and post-treatment cytokine profiles differed significantly more than would be expected by chance (T statistic: -97.01, $p<0.001$, A: 0.396).

The 2-dimensional axes extracted from NMS of GST-specific cytokine responses (Figure 5.6.2) correlated negatively with IL-6 responses (Axis 1) and positively with IL-8 responses (Axis 2). Mean distances differed for both Axis 1 and Axis 2 after treatment reflecting the increase in inflammatory cytokine responses to GST after treatment (Figure 5.1). The difference between pre and post-treatment GST-specific cytokines was confirmed by MRPP (T statistic: -38.60, $p<0.001$, A: 0.114).

Spontaneous cytokine production was also affected by treatment with mean dissimilarities in IL-8 (Axis 1) and IL-21 (Axis 2) after treatment differing to pre-treatment cytokine profiles in un-stimulated whole blood cultures from the same individuals (Figure 5.6.3). However, pre and post-treatment clusters were less distinct than those of CAP, SEA and GST-specific responses, suggesting that the shift was less pronounced than in antigen-specific responses. Dissimilarities between pre and post-treatment cytokine profiles in un-stimulated cultures were significantly greater than those expected by chance (T statistic: -19.61, $p<0.001$, A: 0.042).

In addition to identifying significant difference/dissimilarity between pre and post-treatment responses, NMS also indicates how uniform these treatment-related changes in cytokine profile are within the cohort as indicated by distinct versus overlapping clusters on ordination plot plots (Figure 5.6). Whilst CAP and SEA-specific pre and post-treatment cytokine profiles form distinct clusters in almost all participants (A: 0.205 and 0.396 respectively), overlap between pre and post-treatment cytokine profiles in GST-stimulated (A: 0.114) and un-stimulated cultures (A: 042), suggests that treatment-related changes in GST-specific and spontaneous cytokine responses did not occur in all individuals.

Randomisation tests confirmed that patterns of CAP (Figure 5.6.1), SEA and GST (Figure 5.6.2)-specific cytokine responses and those present in un-stimulated cultures were reliably represented by final 2-dimensional NMS solutions (i.e. relative patterns of pre- and post-treatment cytokine responses were achieved with lower stress than that expected from

randomly assorted data). Consistent with the lack of variation in WWH-specific cytokine responses along axis 2 (Figure 5.6.1), these reliability criteria were met by 1, but not 2-dimensional ordination of WWH-specific cytokines. Diagnostic statistics for all NMS solutions are given in Appendix 3.

5.4.1 Cytokine profiles elicited by cercariae, adult worm and egg antigens 6 weeks post-treatment

Since treatment may alter exposure to the antigens of cercariae, adult worms and eggs differently, I investigated whether the relative cytokine profiles elicited by CAP, WWH and SEA were different 6 weeks post-treatment. Factor analysis was first used to identify CAP, WWH and SEA-specific post-treatment cytokine profiles and extracted 3 PCs. As seen prior to treatment, the majority of variation in cytokine responses was accounted for by inflammatory cytokines (PC1). In addition, PC2 and PC3 were loaded with Th2 and Th17-type cytokine responses (IL-2, IL-4, IL-13, IL-17A and IL-21) and a combination of innate inflammatory and regulatory (IL-8 and IL-10) cytokines respectively. The factor analysis is summarised in Table 5.4.

When regression factor scores for the extracted cytokine profiles were compared by ANOVA (degrees of f, inflammatory (PC1, $F_{2,\ 22}$: 28.081, $p<0.001$) and innate inflammatory/regulatory (PC3, $F_{2,\ 22}$:15.989, $p<0.001$) responses significantly differed according to antigen stimulation. Th2/Th17-type responses (PC2, $F_{2,\ 22}$: 0.608, $p = 0.548$) did not differ according to parasite life-cycle stage. Post-hoc pair-wise comparisons between antigens showed that inflammatory responses were significantly higher in response to egg antigens relative to those of cercariae (T: 5.124, $p<0.001$) or adult worms (T: 7.313, $p<0.001$). Inflammatory factor scores were also significantly higher in response to cercariae than to adult worm antigens (T: 2.189, $p = 0.031$). Mean factor scores for innate inflammatory/regulatory cytokine responses were highest in CAP-stimulated cultures relative to SEA (T: 4.062, $p<0.001$) and WWH (T: 5.438, $p<0.001$). Results of the ANOVA and all post-hoc tests were also significant at the Bonferroni-adjusted significance level. Mean factor scores for each principal component are plotted by antigen in Figure 5.7.

Phenotype	*Cytokine*	*Principal component*		
		1	*2*	*3*
		Inflammatory	*Th2/Th17*	*Innate inflammatory/ Regulatory*
Innate inflammatory	***TNFα***	**0.8**	0.1	0.0
	IL-6	**0.9**	0.1	0.2
	IL-8	**0.6**	-0.1	**0.6**
Th1-type	***IFNγ***	**0.7**	0.1	-0.2
	IL-2	0.0	**0.5**	0.2
	IL-12p70	**0.8**	0.1	-0.3
Th2-type	***IL-4***	-0.2	**0.7**	-0.3
	IL-5	-0.1	0.1	0.0
	IL-10	0.0	0.2	**0.9**
	IL-13	-0.1	**0.6**	0.4
Th17-type	***IL-17A***	-0.2	**0.8**	-0.1
	IL-21	0.0	**0.8**	-0.1
	IL-23	**0.9**	0.1	-0.1
% of total variance		30.3	19.8	11.5

Table 5.4. Factor analysis of life-cycle stage-specific whole blood cytokine responses 6 weeks after treatment. Table shows PCs 1-3 extracted by regression factor analysis and the factor loadings for each of the square-root(x+1)-transformed *S. haematobium* cercariae, adult worm and egg-specific post-treatment cytokine variables. Cytokines with factor loadings ≥0.5 or ≤-0.5 for an extracted PC are highlighted in bold. The cellular immune phenotype with which the cytokines are associated is given for each PC. The percentage of total variance in the dataset accounted for by each PC is given below the relevant column.

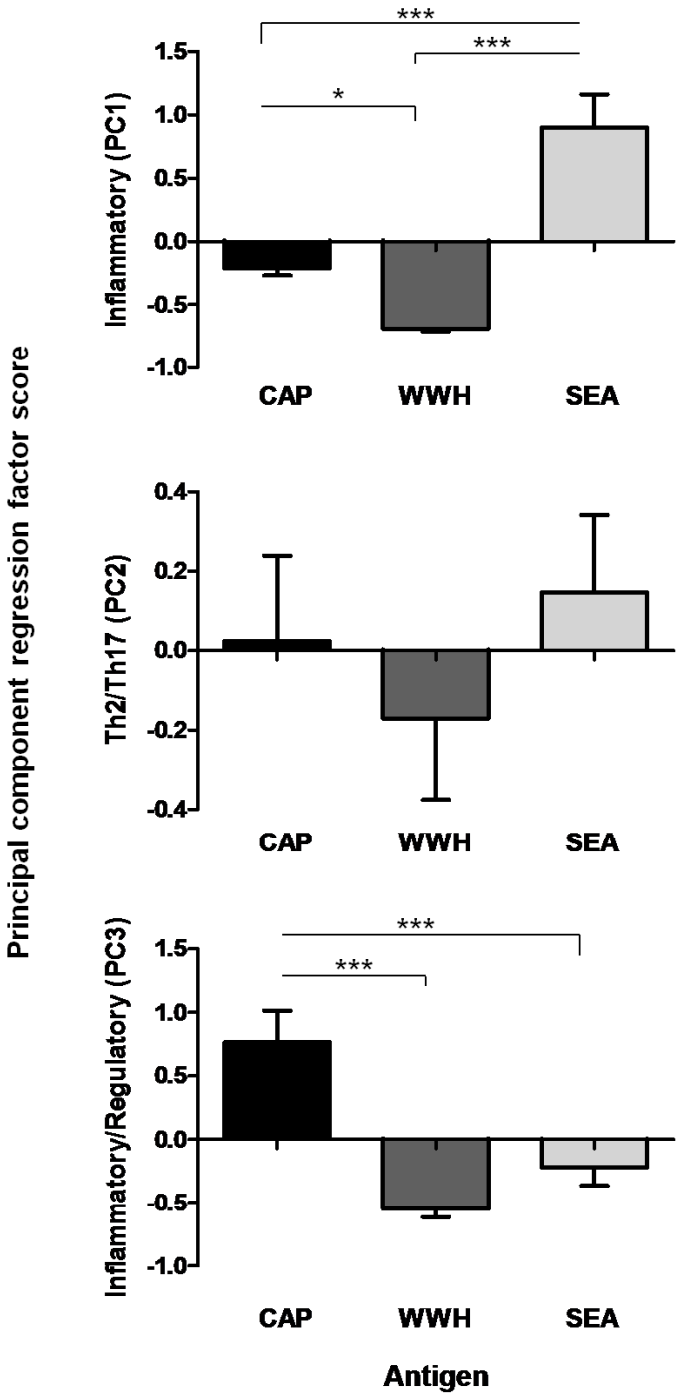


Figure 5.7. Cytokine profiles elicited by antigens of the different *S. haematobium* life-cycle stages 6 weeks post-treatment. Mean regression factor scores for principal components extracted from combined cytokine responses to cercariae (CAP), adult worm (WWH) and egg (SEA) antigens plotted by antigen. Factor scores were compared between antigens by ANOVA. Error bars: standard error of the mean,*p<0.05, ***p<0.001

5.5 Discussion

Praziquantel treatment is known to affect the immune responses to schistosome antigens and may do so either by directly killing parasites and exposing their antigens to immune recognition (Harnett and Kusel 1986; Redman *et al.* 1996) or indirectly by removing immunosuppressive mechanisms mediated by live parasites. Despite many studies investigating pre and post-treatment antibody (Grogan *et al.* 1996b; Mutapi *et al.* 1998a; Mutapi *et al.* 1998b; Mutapi *et al.* 2003; Fitzsimmons *et al.* 2004; Mutapi *et al.* 2005) and cytokine (Roberts *et al.* 1987; van den Biggelaar *et al.* 2002; Fitzsimmons *et al.* 2004; Joseph *et al.* 2004b; Reimert *et al.* 2006) responses to schistosome antigens, none have simultaneously assessed changes in responses to cercariae, adult worm, egg and vaccine-candidate antigens and spontaneous cytokine levels. Furthermore, no previous studies have incorporated changes in innate inflammatory and Th17-type cytokines into their comparison of pre and post-treatment responses.

Comparison of pre and 6 week post-treatment cytokine responses to each antigen identified a dramatic increase in pro-inflammatory cytokine responses to egg antigens and to a lesser extent cercariae and GST. This was evident both from increased levels of TNFα, IL-6, IL-8, IFNγ, IL-2, IL-12p70 and IL-23 relative to pre-treatment levels and from the marked shift towards a more pro-inflammatory cytokine phenotype following treatment. The shift in SEA-specific inflammatory cytokine profiles after treatment was seen in the vast majority of individuals, consistent with a uniform increase in cellular responsiveness to SEA antigens *in vitro*. With the exception of an increase in IL-13, no significant difference in SEA-specific Th2-associated cytokines was observed relative to pre-treatment levels. In contrast, adult worm-specific innate inflammatory (TNFα, IL-6 and IL-8) cytokine responses were lower post-treatment and no significant difference was observed in the post-treatment cytokine profile relative to that observed prior to treatment. Consistent with these observations, egg antigens were found to elicit the most pro-inflammatory cytokine profile of the 3 schistosome life-cycle stages post-treatment. This was in contrast to pre-treatment inflammatory cytokine responses which where highest in response to cercariae (chapter 3).

It is striking that whilst the effects of anti-helminthic treatment are usually reported for adult worms (Becker *et al.* 1980; Hassan *et al.* 1998; Shuhua *et al.* 2000; Gnanasekar *et al.* 2009), the greatest change in whole blood cytokine responses identified in the current study

occurred in response to cercariae and egg antigens. Responses to egg and cercariae may also be boosted by adult worm death since antigens released from dying adult worms upon treatment will contain a cocktail of adult-worm specific antigens and antigens shared with cercariae and eggs (Curwen *et al.* 2004). In particular, death of fecund adult female worms may also increase exposure to immature eggs, which do not have a fully-developed 'shell'(Ashton *et al.* 2001), and thus immune-recognition of somatic egg antigens may be particularly enhanced. The low responsiveness to WWH may be partly due to early and rapid removal of adult worms and their antigens from the circulation following treatment (Hassan *et al.* 1998). In contrast to adult worms, repeated exposure to cercariae during domestic water contacts (Butterworth *et al.* 1985) and prolonged excretion of live and dead eggs trapped in host tissues following treatment (McMahon and Kolstrup 1979; Tchuem Tchuente *et al.* 2004) may mean that the immune system continues to be exposed to the antigens of these life-cycle stages during the 6 weeks following treatment. My observations do not preclude the possibility that cytokine responses to adult worm antigens are 'boosted' at earlier post-treatment timepoints, but indicate that these effects have declined by 6 weeks after treatment. This would be consistent with work in *S. mansoni* identifying a decline in adult worm-specific immune responses within 24 hours of treatment (Fitzsimmons *et al.* 2004) and only short-lived changes in plasma cytokine levels and eosinophil-associated proteins (Reimert *et al.* 2006). *S. haematobium*-specific whole blood cytokine responses also exhibit distinct patterns of secretion within the first 24 hours of treatment that are no longer detectable at 72 hours post-treatment (Scott *et al.* 2000). Whilst these early changes in adult worm-specific cytokine responses are of interest, it is unclear whether they contribute to longer-term changes in the host cytokine environment subsequent to the initial release of antigens from dying worms.

Inflammatory cytokine responses to both cercariae and egg antigens may also increase after treatment due to disruption of immunosuppression mediated by live adult parasites during chronic infection (Maizels and Yazdanbakhsh 2003). This hypothesis is supported by the observation that treatment-induced clearance of adult *S. mansoni* leads to a decline in circulating Treg numbers, suggesting that chronic infection may elevate immunoregulatory responses in the periphery (Watanabe *et al.* 2007). This may be of particular relevance for egg-specific cellular responses, which tend to be high during acute infection and hypo-proliferative in chronically-infected participants prior to treatment (Caldas *et al.* 2000). For example, cytokines such as IL-13 and TNFα, which are associated with egg-induced

immunopathology in mice (Brunet *et al.* 1997; Chiaramonte *et al.* 1999) and humans (Wamachi *et al.* 2004; Martins-Leite *et al.* 2008) and Th1-type cytokines (IFNγ and IL-12p70), which are a feature of acute schistosomiasis in mice (Grzych *et al.* 1991; Pearce and MacDonald 2002) and tend to be lower in chronically-infected than in un-infected humans (Mduluza *et al.* 2003; Silveira *et al.* 2004; Wilson *et al.* 2008), were significantly higher after treatment. Perhaps therefore, after clearance of patent infection by treatment and associated immunoregulation by live parasites, endemically-exposed humans regain the capacity to respond to egg antigens and exhibit a more 'acute' pro-inflammatory cytokine profile. These changes in the SEA-specific effector cytokine profile do not appear to result from changes in the egg-specific regulatory cytokine IL-10, which is not significantly affected by treatment. However, it is possible that alternative mechanisms regulating SEA-specific immune responses may be reduced by treatment. However in the absence of functional assays, it is not possible to draw conclusions about the effect of these changes on the shift in post-treatment cytokine profiles. On-going investigation of the PBMCs collected from the same individuals may help to elucidate how lymphocyte phenotype and schistosome-specific cytokine responses relate to those produced in whole blood culture.

In contrast to SEA, post-treatment increases in CAP-specific IL-8, IL-2, IL-13 and IL-21 responses coincided with an increase in IL-10, which is similar to the association between CAP-specific inflammatory cytokine responses and IL-10 seen before treatment (chapter 3), albeit at elevated concentrations. Repeated exposure to *S. mansoni* cercariae is known to up-regulate both inflammatory and regulatory responses in the skin of mice (Hogg *et al.* 2003b; Cook *et al.* 2011) and fibrotic pathology in baboons (Farah *et al.* 2000) and exposure to infective cercariae between treatment and post-treatment follow-up may partly explain this combined response to CAP in the study cohort. Results from Magaya community suggest that stimulation of both innate inflammatory and regulatory cytokine production is a particular characteristic of *S. haematobium* cercarial antigens, but not WWH and SEA, both before and after treatment. IL-10 derived from innate immune cells appears to be particularly important for the regulation of early inflammatory cytokine responses to cercariae in laboratory mice (Hogg *et al.* 2003b) and this may also be the case after treatment in humans where cercariae-specific inflammatory cytokine responses are elevated and potentially damaging to the host.

It is interesting that the increase in cytokine responsiveness to SEA and CAP was essentially uniform throughout the cohort, suggesting that these changes occurred in all participants regardless of whether they were egg-positive or egg negative at the time of treatment. Thus the change in cytokine responses due to treatment was not solely attributable to the removal of live adult worms. It is possible that parasitological examination may have under-estimated *S. haematobium* and *S. mansoni* infections in the cohort and that cytokine responses were sensitive to removal of these light infections, as has been proposed for *S. haematobium* egg-specific antibody responses (Mutapi *et al.* 1998b). However, even with this possibility in mind, it is unlikely that all participants who responded differently after treatment were infected at the time of treatment. It seems more probable that post-treatment changes in the cytokine responses of participants with no detectable infection may have resulted from the effect of praziquantel on immature parasites present as pre-patent infections or residuals from previous infections (e.g. eggs sequestered in host tissues) (McMahon and Kolstrup 1979; Gnanasekar *et al.* 2009). Although there is evidence for a direct effect of praziquantel on immature schistosomes *in vitro* (Liang et al. 2001) and indirect evidence for an effect after oral administration (Xu *et al.* 1988; Giboda and Smith 1994) it remains un-clear whether treatment alters the host immune response in the absence of adult worms. The relationship between SEA-specific responses and pre-treatment infection intensity is investigated further in chapter 6, but due to the small number of participants who provided sufficient sample volumes for CAP-stimulation this was not possible for cercarial antigens.

The increased levels of inflammatory cytokines and IL-13 elicited by GST after treatment is consistent with studies showing that treatment increases exposure of GST on the adult worm surface (visualised in adult *S. mansoni* from praziquantel treated mice (Dupre *et al.* 1999)) and identification of greater reactivity of serum antibodies to GST isoforms after praziquantel treatment of *S. haematobium*-exposed humans (Mutapi *et al.* 2005). With respect to its potential as an anti-fecundity vaccine targeting adult worms, post-treatment changes in GST-specific cytokine responses are of particular interest since pre-treatment cellular immune responses to *S. haematobium* GST appear to be low (Remoué *et al.* 2001) and high intensity infections are associated with lower levels of GST-specific antibody responses in human population studies (Remoué *et al.* 2000). Thus, the results of the current study suggest that the efficacy of a GST-based vaccine may be enhanced by co-administration with praziquantel and the associated boost in effector cytokine responses I have observed. The benefit of co-administering a GST vaccine with praziquantel has been

previously demonstrated for adult worm-specific vaccination studies in murine infection models (Doenhoff *et al.* 1987; Dupre *et al.* 1999).

Clearance of adult worms after drug-induced paralysis is known to be partially-dependent on immune responses to 'dying worms' (Doenhoff *et al.* 1987) and it is tempting to speculate that GST may be one of several antigens involved in driving this process. However, it is noteworthy that despite the expression of GST by adult parasites (Balloul *et al.* 1985; Curwen *et al.* 2004), cytokine responses to WWH and GST showed distinct patterns following treatment. This is perhaps un-surprising given the variety of other antigens present in WWH; however the large increase in inflammatory cytokine responses to GST, but not to WWH, suggests that GST is not a dominant immunogen of WWH. It is possible that the GST used here, which was purified and expressed in *E. coli*, differ from those present in the WWH but are similar to those present in other life cycle stages (Curwen *et al.* 2004) or lacks the post-translational modifications present in the native protein. Alternatively, cytokine responses to GST present in WWH may peak at earlier time points following treatment (Hassan *et al.* 1998) and those observed 6 weeks after treatment are more reflective of responses to GSTs expressed by larvae and eggs. The relative contributions of different GST isoforms to the cytokine responses elicited by crude antigen preparations could be better assessed in future studies by stimulation of whole blood with equivalent concentrations of purified GST and combinations of isoforms isolated from the different life-cycle stages. This approach might also provide information on whether intra-specific variation between the different parasite strains from which antigens were isolated (i.e. Senegalese and Egyptian) might influence the efficacy of a GST-based vaccine (Galvani 2005).

Although there is considerable precedent for our findings, the elevated whole blood cytokine responses to *S. haematobium* egg antigens after treatment and un-changed responses to adult worm antigens are in marked contrast to some *S. mansoni* studies. For example Roberts and colleagues found that adult worm antigens elicited higher or equivalent cytokine production (TNFα, IFNγ, IL-2, IL-4 and IL-5) to that of egg antigens 3 months after treatment (Roberts *et al.* 1993). Subsequent studies by the same group have also shown very little change in SEA-specific cytokine responses 7 weeks after treatment relative to pre-treatment levels (Joseph *et al.* 2004b). Importantly, these *S. mansoni* studies were conducted in an area of hyper-endemicity and administration of a double dose of praziquantel and the inclusion of participants with persistent infection (20%) in analyses may also have contributed to the elevated adult worm-specific responses relative to my findings (Joseph *et al.* 2004b). The

variation between the findings of human studies provides a strong case for continued investigation in this area in order to elucidate how the efficacy of mass treatment programs implemented in communities with distinct transmission intensities differ and how repeated treatment may influence immune post-treatment relative to a single anti-helminthic dose. The latter has been investigated in *S. haematobium* using treatment at 3 month intervals, showing that SEA-specific antibody responses to SEA differed (higher IgE and lower IgG4) in children who received both single and repeated doses relative to un-treated children and particularly so after multiple treatments, however SEA-specific cytokine responses were not investigated (van den Biggelaar *et al.* 2002). Consistent with the observations of my study of whole blood cytokine responses, PBMC Th2-type cytokine responses were not significantly higher than un-treated children 3 months after a single dose of praziquantel but were higher in repeatedly treated children (van den Biggelaar *et al.* 2002).

Due to the high intensity of infections in Magaya community all participants received treatment and thus it was not possible to compare post-treatment cytokine responses to an un-treated control group. Thus, whilst the follow-up study was conducted within weeks of initial sampling and there were no obvious environmental or behavioural changes during this period, the possibility that differences in the cytokine profiles measured are due to temporal changes in cercarial exposure in the study cohort cannot be excluded. However, exclusion of non-permanent residents of the area and conducting both pre and post-treatment surveys during the dry season minimised this possibility. Furthermore, the use of identical culture conditions and antigen preparations in the field and ELISA reagents and protocols for subsequent cytokine assays mean that methodological differences between pre and post-treatment cytokine responses are highly unlikely.

5.6 Conclusions

The results of the current chapter suggest firstly that the antigens of *S. haematobium* cercariae, adult worms and eggs continue to elicit distinct cytokine profiles following treatment with praziquantel. Furthermore, a single dose of anti-helminthic is associated with dramatic changes in the parasite-specific cytokine responses of endemically-exposed participants that persist for up to 6 weeks after treatment. These treatment-related changes are predominantly due to an increase in inflammatory cytokines to egg antigens and to a lesser extent cercarial antigens and the vaccine candidate antigen GST. When considered

together, the cytokine profiles elicited by adult worm antigens after treatment were not significantly altered relative to pre-treatment profiles in the same cohort.

Thus, treatment induced changes in the cytokine responses to *S. haematobium* have potential relevance to the development of resistance to subsequent re-infection, as explored in chapter 6. In particular the increased cytokine response to GST after treatment is consistent with studies identifying increased exposure of this antigen after treatment and the potential for treatment to boost the efficacy of a GST-based anti-schistosome vaccine. The latter observation supports current recommendations that schistosome vaccine strategies should incorporate a treatment aspect.

Chapter 6

Relating changes in post-treatment cytokine profiles to pre-treatment *Schistosoma haematobium* infection intensity and the risk of re-infection

6.1 Introduction

Treatment-re-infection studies have identified an association between parasite-specific antibody titres (Hagan *et al.* 1991; Mutapi *et al.* 1999; Caldas *et al.* 2000) and cellular proliferative responses (Colley *et al.* 1986) and a reduced risk of re-infection. However, the association between cytokine responses and the risk of re-infection is more controversial (Roberts *et al.* 1993; Medhat *et al.* 1998; van den Biggelaar *et al.* 2002) due to inconsistencies in study populations and cytokines assayed in studies conducted to-date (chapter 1.8.3).

In chapter 5 I showed that treatment of Magaya community lead to a change in parasite-specific cytokine responses relative to pre-treatment levels and the initial aim of this chapter is to address how host characteristics and infection levels prior to treatment contribute to variation in the magnitude of this change. In particular, since the effect of treatment on parasite-specific immune responses is related to exposure of parasite antigens (Harnett and Kusel 1986; Dupre *et al.* 1999) and/or removal of immunosuppression by live parasites (Woolhouse and Hagan 1999), I hypothesised that post-treatment changes would be influenced by pre-treatment parasite density (i.e. infection intensity).

Using longitudinal data on re-infection within Magaya community I subsequently investigate whether post-treatment cytokine profiles affect the risk of re-infection within 18 months of treatment. As in previous chapters I have assayed a panel of 13 cytokines to avoid inferring the schistosome-specific cellular immune polarisation from a limited sub-set of biomarkers. Furthermore, I have focused on adult worm and egg-specific cytokine responses, because these stages of the schistosome life-cycle are most closely related to infection intensity quantified via urine egg counts, and because both are simultaneously present during

infection. This is also the first study to investigate whether post-treatment cytokine responses to the vaccine candidate glutathionine-S-transferase (GST) correspond to a reduced risk of re-infection.

6.2 Hypotheses

- Pre-treatment infection intensity influences the magnitude of change in parasite-specific cytokine responses 6 weeks after treatment

- *S. haematobium*-specific cytokine profiles 6 weeks after treatment influence the risk of re-infection within 18 months of treatment

6.3 Materials and Methods

6.3.1 Study participants

This aspect of the study includes the same cohort of participants as chapter 5 (n = 94). These participants were included according to the following criteria:1) provided a minimum of 2 urine samples before and after treatment for quantification of *S. haematobium* infection, 2) were negative for co-infections (*S. mansoni*, STH, malaria and HIV) before and after treatment, 3) had received a single dose of praziquantel (40mg/kg) 6 weeks earlier (but had not received treatment for schistosomiasis prior to recruitment), 4) were successfully cleared of *S. haematobium* infection or remained un-infected 6 weeks after treatment (defined as no eggs detectable in urine samples), 5) were life-long permanent residents of the study area and 6) provided a venous blood sample of sufficient volume to conduct whole blood cultures both before and 6 weeks after treatment. The demographic and pre-treatment infection characteristics of the participants followed up post-treatment are summarised in Table 6.1.

	Age group (years)		
	5 - 10	***11 – 12***	***13+***
n	53	18	23
Mean age	7.6	11.6	30.4
Number of males: females	32:21	7:11	11:12
Mean pre-treatment infection intensity*(S.E.M.)	31.52 (12.45)	59.85 (32.00)	10.35 (4.29)
Pre-treatment infection range*	0 - 481	0 - 502	0 - 91
Pre-treatment infection prevalence (%)	52.8	61.1	56.5

Table 6.1. Demographic and pre-treatment *S. haematobium* infection characteristics of each age group included in 6 weeks post-treatment cytokine analysis. *Infection intensity quantified as mean egg counts/10ml urine. S.E.M. – standard error of the mean.

The relationship between 6 weeks post-treatment cytokine responses and re-infection status 18 months after treatment was investigated in 53 of the individuals who met the above inclusion criteria and provided follow-up samples for parasitological analyses at subsequent

sampling visits. Of these individuals, 43 were followed-up 6 months post-treatment and all provided samples 18 months post-treatment. The characteristics of the participants included in the re-infection study are summarised in Table 6.2.

	Age group (years)		
	5 - 10	***11 – 12***	***13+***
n	38	10	5
Mean age	7.6	11.6	13.4
Number of males: females	23:15	4:6	4:1
Mean pre-treatment infection intensity*(S.E.M.)	34.41 (16.32)	21.70 (13.79)	39.75 (19.09)
Pre-treatment infection range*	0 - 481	0 - 137	0 - 91.33
Number of re-infected participants **	11	3	1

Table 6.2. Demographic and pre-treatment *S. haematobium* infection characteristics of each age group included in 18 months re-infection analysis. *Infection intensity quantified as mean egg counts/10ml urine. S.E.M. – standard error of the mean. **Re-infection classified as presence of one or more *S. haematobium* eggs in urine at 6 months or 18 months post-treatment.

6.3.2 Immunological assays

Whole blood samples collected 6 weeks after treatment were cultured for 48 hours with 10μg/ml *S. haematobium* whole worm homogenate (WWH), soluble egg antigen (SEA), 2 μg/ml GST or left un-stimulated as controls for spontaneous cytokine production. Parallel PHA-stimulated (2μg/ml) cultures acted as positive controls, indicating the viability and potential of cultured whole blood cells to secrete cytokines in response to non-parasite antigens.

Innate inflammatory (TNFα, IL-6 and IL-8), Th1 (IFNγ, IL-2 and IL-12p70), Th2 (IL-4, IL-5, IL-10 and IL-13) and Th17 (IL-17A, IL-21 and IL-23)-type cytokines were assayed in culture supernatants via enzyme-linked immunosorbent assay (ELISA) as described in Chapter 2.3.5.5.

6.3.3 Statistical Analyses

The change in cytokine responses to adult worm and egg antigens after treatment was obtained by subtracting pre-treatment cytokine levels (un-transformed) from 6 weeks post-treatment cytokine levels (un-transformed). It was considered important to assess the magnitude of change relative to pre-treatment levels rather than post-treatment cytokine responses alone to account for the existing variation between participant cytokine responses observed before treatment (chapter 3). These changes were then grouped by factor analysis to identify principal components (PCs) corresponding to patterns of treatment-related changes in cytokine responses to *S. haematobium* antigens. High regression factor scores for extracted components correspond to a greater change in cytokine responses positively associated with the component (factor score $\geq$0.5) and a lesser change in cytokine responses negatively associated with the component (factor score $\leq$ -0.5)) relative to pre-treatment responses. All WWH and SEA-specific cytokine changes were included in the same analysis as separate factors so that any differences in SEA and WWH-specific responses would also be characterised by the resulting PCs. A separate factor analysis was conducted for the change in GST-specific cytokine responses after treatment.

To address the hypothesis that treatment-related changes in cytokine profiles were dependent on pre-treatment infection levels, factor scores for each extracted PC (i.e. the cytokine profiles most affected by treatment) were analysed as dependent variables via analysis of variance (ANOVA). Sequential sums of squares were used to account for variation due to sex and age group (5-10, 11-12 and 13+ years). Identification of age groups according to the age range at which infection intensity is increasing (5-10 years), peaking (11-12 years) and declining (13+ years) within Magaya community is described in chapter 2.3.7. The interaction between age group and pre-treatment infection intensity was included in the ANOVA to identify whether the effect of pre-treatment infection intensity was age-dependent. Exploratory analysis verified that regression factor scores for all PCs and residuals of ANOVA models met parametric assumptions. Post-hoc analysis of the correlation between the residuals of pre-treatment infection intensity and the residuals of the PC factor scores after accounting for age group and sex was conducted using Pearson's 2-tailed correlation procedure. Where the interaction between pre-treatment infection intensity and age group was found to significantly affect the change in cytokine profiles, Pearson's correlation analysis was also conducted separately for each age group to investigate how age

influenced the relationship between the residual variation in post-treatment changes in host cytokine profiles and the residual variation in pre-treatment infection intensity after accounting for variation due to age and sex.

To characterise the cytokine profiles that result from treatment square root(x+1)-transformed 6 weeks post-treatment cytokine responses were reduced into a smaller number of variables by factor analysis. This analysis only included the cytokine responses of participants who provided samples for parasitological analysis 18 months after treatment (n = 53). WWH and SEA-specific cytokine responses 6 weeks post-treatment were entered into a single factor analysis and a separate analysis was conducted for GST-specific responses.

Binary logistic regression was subsequently used to investigate whether *S. haematobium*-specific 6 weeks post-treatment cytokine profiles were significant predictors of re-infection within 18 months of treatment. I chose to investigate the effect of post-treatment cytokine responses, rather than pre-treatment responses as investigated in previous studies (Hagan *et al.* 1991; Mutapi *et al.* 1999; Caldas *et al.* 2000), since re-infection occurs in the context of treatment-altered immune responses. Binary logistic regression was conducted for WWH and SEA-specific cytokine profiles and separately for GST-specific cytokine profiles The dependent variable was re-infection status and individuals were categorised as 're-infected' if they had 1 or more *S. haematobium* eggs detectable in any of their urine samples 6 months or 18 months post-treatment or 'un-infected' if they were egg negative at both post-treatment timepoints. Sex (male, female), age (covariate), pre-treatment infection intensity (covariate) and all adult worm and egg-specific cytokine profiles at 6 weeks post-treatment (i.e. 6 week post-treatment PC factor scores) were included as predictors in the final model so that each was analysed holding all other potential predictors constant. The Wald X^2 statistic was used to assess whether the coefficient (B) of each linear predictor in the model was greater than 0 (Sokal and Rohlf 1995d) and predictors were considered to contribute significantly to the risk of re-infection if $p<0.05$. Hosmer Lemeshow (H-L) tests, which compare model predictions with the original re-infected/and un-infected data, were used to determine whether regression models appropriately fit the original data. Models were considered an acceptable fit when the significance of the associated H-L test statistic was >0.05. For significant predictors of re-infection status, mean scores of cytokine profiles were compared between un-infected and re-infected groups by post-hoc un-paired, 2-tailed Student's t-test.

Results of each set of comparisons were corrected for multiple comparisons using the sequential Bonferroni method (Holm 1979; Rice 1989) and results significant after correction are indicated alongside raw p-values.

6.4 Results

6.4.1 The effect of pre-treatment infection intensity on the change in adult worm and egg-specific cytokine profiles 6 weeks post-treatment

Changes in WWH and SEA-specific cytokine responses 6 weeks after treatment were grouped into 4 PCs (Table 6.3). Unlike factor analysis of pre-treatment cytokine responses, for which SEA and WWH-specific responses grouped into similar cytokine profiles (chapter 4), post-treatment changes in SEA- and WWH-specific cytokine responses loaded onto distinct PCs. The latter reflects the large increase in cytokine responses to SEA, but less pronounced changes in WWH-specific cytokine responses post-treatment (chapter 5).

The greatest change in cytokine responses due to treatment was seen in PC1, which corresponded to a mixed profile of egg-specific cytokines including innate inflammatory (IL-6), Th1 (IFNγ and IL-12p70), Th2 (IL-4) and Th17 (IL-23)-type cytokines. The change in IL-4 responses to adult worm antigens was also significantly loaded onto this component (PC1). PC2 reflected changes in all adult-worm-specific Th17-type cytokines (IL-17A, IL-21 and IL-23) and was negatively correlated with changes in egg-specific TNFα and IL-13. Changes in adult worm-specific innate (IL-6 and IL-8), Th1 (IFNγ) and Th17 (IL-23)-type pro-inflammatory cytokines were grouped in PC3. PC4 was negatively correlated with changes in adult worm and egg-specific IL-21.

Factor scores for the post-treatment change in mixed cytokine responses to egg antigens (PC1) and egg and adult worm-specific IL-21 (PC4) were significantly affected by pre-treatment infection intensity (not significant after Bonferroni correction). Post-hoc Pearson's correlation analysis indicated that the relationship was positive for both PC1 and PC4. The positive correlation between pre-treatment infection intensity and PC4 is indicative of a negative correlation with the change in IL-21 responses to adult worm and egg antigens, which are negatively loaded onto the component. Thus, whilst participants with high pre-treatment infection intensity had a greater increase in PC1-type responses, treatment lead to lesser changes in PC4-type responses in these individuals. These patterns were independent of host age group and the interaction between host age group and pre-treatment infection intensity. Factor scores for each component are plotted against pre-treatment infection intensity in Figure 6.1. Results of ANOVA and post-hoc tests are summarised in Table 6.4.

			Principal component			
			1	***2***	***3***	***4***
Phenotype	***Cytokine***	***Antigen***	SEA mixed	WWH Th17	WWH inflammatory	IL-21
Innate inflammatory	***ΔTNFα***	***SEA***	0.4	**-0.5**	0.1	-0.3
		WWH	0.2	0.4	**0.7**	0.2
	ΔIL-6	***SEA***	**0.7**	-0.2	0.4	-0.1
		WWH	-0.1	0.3	**0.6**	-0.1
	ΔIL-8	***SEA***	-0.1	-0.2	0.2	-0.1
		WWH	0.0	0.4	0.0	-0.3
Th1-type	***ΔIFNγ***	***SEA***	**0.5**	-0.3	0.1	-0.3
		WWH	0.0	0.3	**0.5**	0.3
	ΔIL-2	***SEA***	-0.2	-0.1	0.1	0.4
		WWH	-0.2	0.2	-0.1	0.3
	ΔIL-12p70	***SEA***	**0.6**	-0.3	0.3	0.1
		WWH	0.0	0.3	0.3	0.2
Th2-type	***ΔIL-4***	***SEA***	**0.5**	0.1	-0.3	0.4
		WWH	**0.5**	0.1	-0.1	0.3
	ΔIL-5	***SEA***	0.3	0.0	-0.2	0.4
		WWH	0.4	0.0	-0.2	0.4
	ΔIL-10	***SEA***	-0.3	0.0	0.3	-0.2
		WWH	-0.3	-0.1	0.2	-0.3
	ΔIL-13	***SEA***	-0.4	**-0.5**	0.1	0.3
		WWH	-0.4	-0.4	0.1	0.3
Th17-type	***ΔIL-17A***	***SEA***	0.3	0.4	-0.2	0.2
		WWH	0.4	**0.5**	-0.2	0.0
	ΔIL-21	***SEA***	0.0	0.3	-0.2	**-0.5**
		WWH	-0.1	**0.5**	-0.3	**-0.5**
	ΔIL-23	***SEA***	**0.6**	-0.4	0.3	-0.3
		WWH	0.1	**0.5**	**0.5**	0.1
% of total variance			12.2	10.3	9.4	8.4

Table 6.3. Factor analysis of the change in adult worm and egg-specific cytokine responses 6 weeks post-treatment relative to pre-treatment levels (n = 94). Table shows PCs 1-4 extracted by regression factor analysis and the factor loadings for the difference (Δ) between post-treatment and pre-treatment cytokine responses elicited by *S. haematobium* whole worm homogenate (WWH) and soluble egg antigen (SEA). Cytokines with factor loadings ≥0.5 or ≤-0.5 for an extracted PC are highlighted in bold. The cellular immune phenotype with which the cytokines are associated is given for each PC. The percentage of total variance in the dataset accounted for by each PC is given below the relevant column.

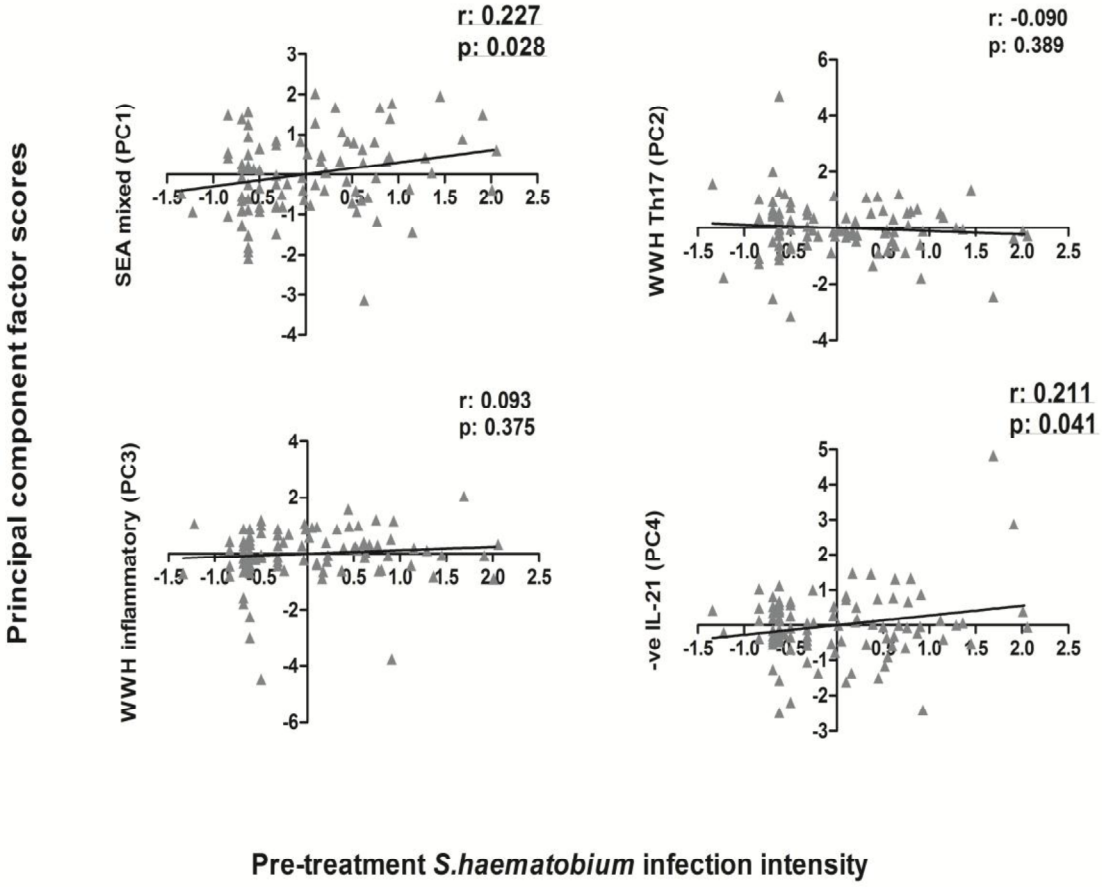


Figure 6.1. Post-treatment changes in a mixed profile of egg-specific cytokine responses (PC1) and in adult worm and egg-specific IL-21 (PC4) are associated with pre-treatment infection intensity (n = 94). Residual variation in PC factor scores for each cytokine profile after accounting for sex and age group are plotted against residuals of pre-treatment infection intensity (log10(mean egg counts/10ml urine +1)-transformed). Pearson's r and the associated p-value are given to indicate direction and significance of the correlation. Significant correlations are highlighted in bold and under-lined. **–** ve IL-21**–** IL-21 is negatively associated with PC4 and therefore higher PC4 factor scores correspond to a lesser change in IL-21 responses

Factor$_{(degrees\ of\ freedom)}$	*SEA mixed (PC1)*			*WWH Th17 (PC2)*			*WWH inflammatory (PC3)*			*-ve IL-21 (PC4)*		
	F	***p***	***Post-hoc comparison***	***F***	***p***	***Post-hoc comparison***	***F***	***p***	***Post-hoc comparison***	***F***	***p***	***Post-hoc comparison***
Sex$_{(1,87)}$	0.367	0.546		0.167	0.684		2.796	0.098		1.044	0.310	
Age group$_{(2,87)}$	0.682	0.508		0.492	0.613		0.506	0.605		1.189	0.309	
Pre-treatment infection intensity$_{(1,87)}$	4.664	**0.034**	**+ve correlation**	0.442	0.508		0.549	0.461		6.910	**0.010**	**+ve correlation**
Age group* Infection intensity$_{(2,87)}$	0.568	0.568		1.925	0.152		1.091	0.340		0.269	0.765	

Table 6.4. Changes in adult worm and egg-specific cytokine profiles after treatment are dependent on pre-treatment infection intensity (n = 94). Results of ANOVA analysis of the change in cytokine profiles 6 weeks post-treatment are summarised and significant effects ($p<0.05$) are highlighted in bold. No comparisons were significant after Bonferroni correction for multiple comparisons. – ve IL-21– IL-21 is negatively associated with PC4 and therefore higher PC4 factor scores correspond to a lesser change in IL-21 responses

6.4.2 The effect of pre-treatment infection intensity on the change in GST-specific cytokine profiles 6 weeks post-treatment

Factor analysis of the change in GST-specific cytokine responses extracted 3 cytokine profiles associated with the greatest magnitude of change post-treatment. PC1 was loaded with changes in inflammatory cytokine responses (TNFα, IL-6, IL-12p70 and IL-23) and negatively loaded with the change in IL-10, indicating that individuals with large changes in pro-inflammatory cytokines after treatment had less marked changes in IL-10. PC2 had a positive loading with changes in IL-2 and IL-13, but a negative loading with changes in IL-4. PC3 reflected changes in both innate inflammatory (IL-8) and Th17 (IL-21)-associated cytokines. The factor analysis is summarised in Table 6.5.

GST-specific inflammatory cytokine responses (PC1) were not significantly correlated with pre-treatment infection intensity alone, but were significantly influenced by the interaction between host age group and pre-treatment infection intensity (not significant after Bonferroni correction). Correlation analysis of this relationship by age group indicated that the change in GST-specific inflammatory cytokines was significantly negatively correlated with pre-treatment infection intensity in 11-12 year olds, in whom infection intensity is peaking. Interestingly 5-10 year olds, in whom infection intensity is increasing, showed the opposite relationship, although this trend was not statistically significant.

Changes in PC2, reflecting a greater change in IL-2 and IL-13 and lesser change in IL-4, were significantly negatively correlated with pre-treatment infection intensity, although the effect of infection intensity was not significant after Bonferroni correction for multiple comparisons. This relationship was independent of host age group and sex.

PC3 was not significantly affected by sex, age group, pre-treatment infection intensity or the interaction between age group and pre-treatment infection intensity.

Factor scores for each GST-specific PC are plotted in Figure 6.2 and GST-specific inflammatory cytokine responses (PC1) are plotted by infection intensity for each age group in Figure 6.3. Statistical analyses are summarised in Table 6.6.

			Principal Components		
			1	***2***	***3***
Phenotype	***Cytokine***	***Antigen***	Inflammatory	IL-2,IL-13/ IL-4	Innate inflammatory/ Th17
Innate inflammatory	***ΔTNFα***	***GST***	**0.8**	0.1	0.3
	ΔIL-6	***GST***	**0.6**	0.1	**0.5**
	ΔIL-8	***GST***	0.1	0.4	0.1
Th1-type	***ΔIFNγ***	***GST***	0.4	-0.1	0.4
	ΔIL-2	***GST***	0.0	**0.5**	-0.2
	ΔIL-12p70	***GST***	**0.6**	-0.2	-0.2
Th2-type	***ΔIL-4***	***GST***	-0.1	**-0.5**	0.2
	ΔIL-5	***GST***	-0.1	-0.2	0.3
	ΔIL-10	***GST***	**-0.6**	0.3	0.4
	ΔIL-13	***GST***	0.2	**0.7**	-0.3
Th17-type	***ΔIL-17A***	***GST***	-0.2	-0.4	0.2
	ΔIL-21	***GST***	-0.3	0.4	**0.6**
	ΔIL-23p19	***GST***	**0.6**	0.0	0.1
% of total variance			18.7	12.6	10.6

Table 6.5. Factor analysis of the change in GST-specific cytokine responses 6 weeks post-treatment relative to pre-treatment levels (n = 94). Table shows principal components (1-3) extracted by regression factor analysis and the factor loadings for the difference (Δ) between post-treatment and pre-treatment cytokine responses elicited by *S. haematobium* GST. Cytokines with factor loadings ≥0.5 or ≤-0.5 for an extracted PC are highlighted in bold. The cellular immune phenotype with which cytokines are associated is given for each PC. The percentage of total variance in the dataset accounted for by each PC is given below the relevant column.

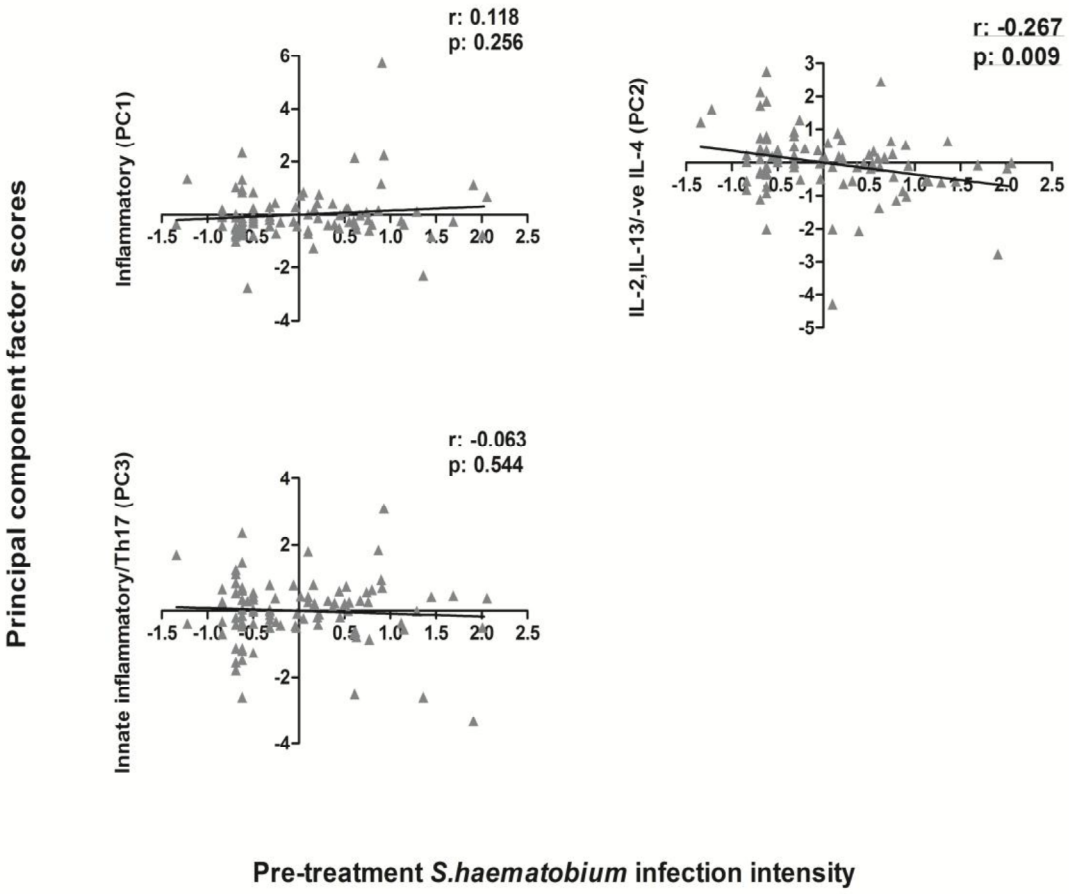


Figure 6.2. Variation in the magnitude of change in GST-specific IL-2, IL-13/ IL-4 responses (PC2) after treatment is associated with pre-treatment infection intensity (n = 94). Residual variation in factor scores for each cytokine profile after accounting for sex and age group are plotted against residual variation in pre-treatment infection intensity (log10(mean egg counts/10ml urine+1)-transformed). Pearson's r and the associated p-value are given to indicate direction and significance of the correlation. Significant correlations are highlighted in bold and under-lined. –ve IL-4 - IL-4 is negatively associated with PC2 and therefore higher PC2 factor scores correspond to a lesser change in IL-4 responses

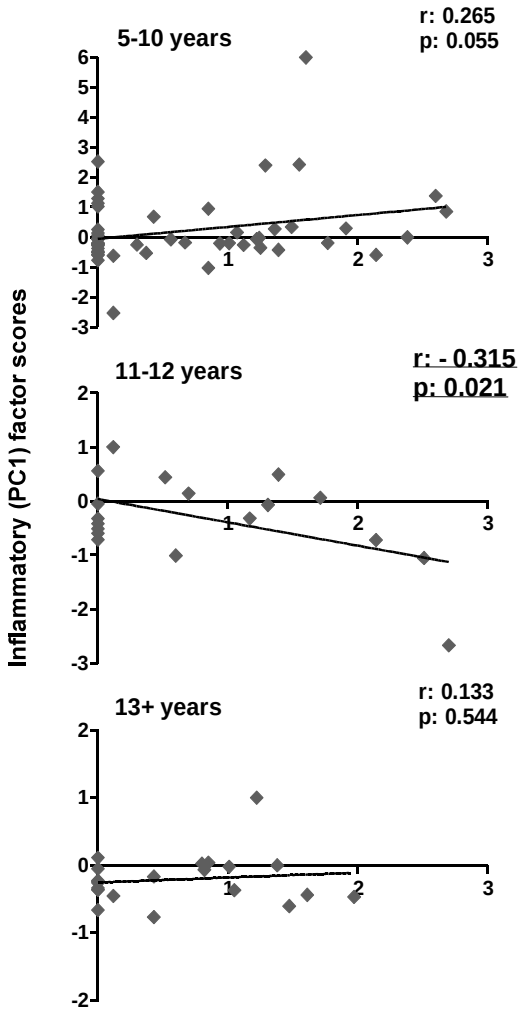


Figure 6.3. The increase in GST-specific inflammatory cytokine responses is negatively correlated with pre-treatment infection intensity in 11-12 year olds. Factor scores for PC1, which corresponds to TNFα, IL-6, IL-12p70 and IL-23 responses, are plotted against pre-treatment infection intensity (mean egg counts/10ml urine) for each age group. Since PC1 is negatively associated with IL-10, high factor scores reflect a lesser change in IL-10. Pearson's r and the associated p-value are given to indicate direction and significance of the correlation within each age group. Significant correlations are highlighted in bold and under-lined.

Factor *(degrees of freedom)*	*Inflammatory (PC1)*			*IL-2,IL-13/-ve IL-4 (PC2)*			*Innate inflammatory/Th17 (PC3)*		
	F	***p***	***Post-hoc comparison***	***F***	***p***	***Post-hoc Comparison***	***F***	***p***	***Post-hoc comparison***
Sex *(1,87)*	0.005	0.945		1.394	0.241		0.366	0.547	
Age group *(2,87)*	2.754	0.069		1.606	0.207		0.424	0.656	
Infection intensity *(1,87)*	1.153	0.286		5.338	**0.023**	**-ve correlation**	0.715	0.400	
Age group*Infection intensity *(2,87)*	3.737	**0.028**		0.462	0.631		0.970	0.383	

Table 6.6. Changes in GST-specific cytokine profiles after treatment are dependent on pre-treatment infection intensity and age group (n = 94). Results of ANOVA analysis of the change in cytokine profiles 6 weeks post-treatment are summarised and significant effects ($p<0.05$) are highlighted in bold. Those significant after Bonferroni correction for multiple comparisons are shaded grey.

6.4.4 Re-infection 6 months and 18 months post-treatment

Of the 53 individuals included in the longitudinal post-treatment follow-up of *S. haematobium* infection levels, 15 were re-infected within 18 months of infection clearance by treatment. Infection intensity and prevalence of *S. haematobium* eggs in urine before, 6 weeks, 6 months and 18 months post-treatment are shown in Figure 6.4.

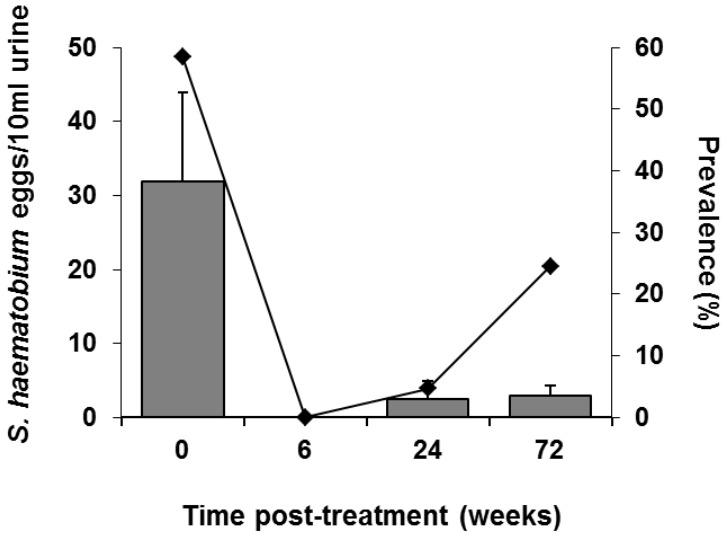


Figure 6.4. *S. haematobium* infection intensity and prevalence before and 6 weeks, 6 months and 18 months after praziquantel treatment. Bar chart shows mean infection intensity of the cohort prior to treatment (n = 53) and at each follow-up visit post-treatment: 6 (n = 53), 24 (n =43) and 72 (n = 53) weeks. Black line indicates change in infection prevalence over time. Error bars: standard error of the mean.

6.4.4 Adult worm and egg-specific cytokine profiles 6 weeks post-treatment and the risk of re-infection

Adult worm and egg-specific cytokine responses at 6 weeks post-treatment for the 53 individuals with re-infection data were grouped into 7 PCs which reflected distinct patterns of responses to adult worm and egg-stage parasite antigens. The factor analysis is summarised in Table 6.7.

			Principal components						
			1	***2***	***3***	***4***	***5***	***6***	***7***
Phenotype	***Cytokine***	***Antigen***	SEA Th1/Th2 & WWH mixed	IL-21/ SEA inflamm	WWH innate inflamm/ Th17	Th2/ Th17	SEA Th2/ WWH Th1	SEA innate inflamm/ Th2	SEA innate Inflamm/ WWH Th1
Innate inflamm.	***TNFα***	***SEA***	**-0.5**	0.4	0.0	-0.3	0.1	**0.5**	-0.1
		WWH	**0.5**	0.3	0.1	-0.1	0.0	0.0	-0.4
	IL-6	***SEA***	**-0.6**	**0.7**	0.0	0.0	0.3	0.1	0.0
		WWH	-0.1	0.2	**0.8**	0.2	0.1	0.1	0.3
	IL-8	***SEA***	-0.2	0.2	-0.2	-0.4	-0.2	0.0	**0.5**
		WWH	-0.1	0.2	**0.8**	0.3	0.2	0.0	0.3
Th1-type	***IFNγ***	***SEA***	-0.2	**0.6**	-0.2	-0.4	0.0	-0.1	-0.3
		WWH	0.2	0.3	-0.2	-0.2	-0.1	**-0.6**	**0.5**
	IL-2	***SEA***	**0.7**	0.1	0.1	-0.1	0.2	-0.3	0.0
		WWH	**0.8**	0.1	0.1	0.0	0.2	0.1	-0.2
	IL-12	***SEA***	-0.3	**0.7**	0.0	-0.2	0.0	-0.3	0.1
		WWH	0.4	0.1	0.2	-0.2	**0.5**	-0.1	-0.1
Th2-type	***IL-4***	***SEA***	0.2	0.1	-0.4	**0.5**	**0.6**	0.1	0.1
		WWH	0.1	0.1	-0.4	**0.6**	0.3	0.0	0.1
	IL-5	***SEA***	0.0	0.2	-0.4	0.4	**0.5**	0.1	0.2
		WWH	-0.1	0.4	-0.4	0.3	0.2	0.1	-0.1
	IL-10	***SEA***	**0.7**	0.1	0.0	-0.3	0.0	0.3	0.2
		WWH	**0.6**	0.3	0.1	**-0.5**	0.3	-0.2	-0.2
	IL-13	***SEA***	**0.6**	0.1	-0.1	-0.4	0.0	**0.5**	0.3
		WWH	**0.7**	0.1	0.0	-0.2	-0.1	0.4	0.2
Th17-type	***IL-17A***	***SEA***	0.3	0.4	0.4	**0.5**	-0.4	0.1	0.0
		WWH	0.4	0.3	0.2	**0.5**	**-0.5**	0.1	-0.2
	IL-21	***SEA***	0.2	**0.5**	-0.3	0.2	-0.3	-0.3	0.0
		WWH	**0.5**	**0.5**	-0.2	0.3	-0.4	0.0	-0.1
	IL-23	***SEA***	**-0.5**	**0.6**	0.0	-0.2	0.0	0.3	-0.1
		WWH	0.1	0.2	**0.7**	0.0	0.3	-0.2	-0.1
% of total variance			19.2	12.9	11.1	9.9	7.6	6.0	5.4

Table 6.7. Factor analysis of adult worm and egg-specific cytokine responses at 6 weeks post-treatment (n = 53). Table shows PCs 1-7 extracted by regression factor analysis and the factor loadings for post-treatment cytokine responses elicited by *S. haematobium* whole worm homogenate (WWH) and soluble egg antigen (SEA). Cytokines with factor loadings ≥0.5 or ≤-0.5 for an extracted PC are highlighted in bold. The cellular immune phenotype with which the cytokines are associated is given for each PC. The percentage of total variance in the dataset accounted for by each PC is given below the relevant column.

inflamm - inflammatory

Having characterised the main post-treatment cytokine profiles mounted in response to WWH and SEA *in vitro*, I investigated whether these responses were related to the risk of subsequent re-infection. Binary logistic regression of re-infection status within 18 months of treatment holding other potential risk factors for re-infection (sex, age group, pre-treatment infection intensity and all other principal components) constant indicated that factor scores for IL-21 and SEA-specific inflammatory cytokine responses (PC2) were a significant predictor of re-infection status (Wald X^2: 4.515, p = 0.034, H-L X^2: 12.12, p = 0.146) however this effect was not significant when significance levels were adjusted for multiple comparisons (sequential Bonferroni correction). The odds ratio indicates that for each 1 point increase in PC2 factor scores, indicating a heightened PC2-type response to schistosome antigens, there was a reduction in the risk of re-infection by a factor of 0.37. The results of the analyses are summarised in Table 6.8.

Post-hoc t-test comparison of PC2 factor scores between un-infected individuals and those re-infected within 18 months of treatment confirmed that IL-21 and SEA-specific inflammatory responses were higher in participants who remained un-infected than those who were re-infected (t_{51}: 2.151, p = 0.036). Sex, age group, pre-treatment infection intensity and the other adult worm and egg-specific cytokine profiles at 6 weeks post-treatment were not significant predictors of re-infection. PC2 factor scores are plotted by re-infection status in Figure 6.5.

Predictor	*B*	*Wald X^2*	*p*	*Odds Ratio*	*95% CI*
Sex	0.227	0.096	0.757	1.255	0.30 - 5.28
Age	-0.212	1.227	0.268	0.809	0.56 - 1.18
Pre-treatment infection intensity	0.004	0.873	0.350	1.004	1.00 - 1.01
SEA & WWH-specific cytokine profile:					
SEA Th1/Th2 & WWH mixed (PC1)	0.349	0.933	0.334	1.418	0.70 - 2.88
IL-21 & SEA inflammatory (PC2)	-1.194	5.570	**0.018**	.303	0.11 - 0.82
WWH inflammatory (PC3)	0.233	0.199	0.655	1.262	0.45 - 3.51
Th2/Th17 (PC4)	0.229	.292	0.589	1.257	0.55 - 2.89
SEA Th2 & WWH Th1 (PC5)	0.305	0.764	0.382	1.357	0.69 - 2.69
SEA innate inflammatory/Th2 (PC6)	0.570	1.218	0.270	1.768	0.64 - 4.86
SEA innate inflammatory & WWH Th1 (PC7)	-0.845	2.232	0.135	0.430	0.14 - 1.30

Table 6.8. Adult worm and egg-specific cytokine responses at 6 weeks post-treatment influence the risk of re-infection (n = 53). Results of binary logistic regression analysis of participant re-infection status within 18 months of praziquantel treatment. Significant predictors of re-infection ($p<0.05$) are highlighted in bold. B – beta coefficient, 95% CI – 95% confidence interval for odds ratio.

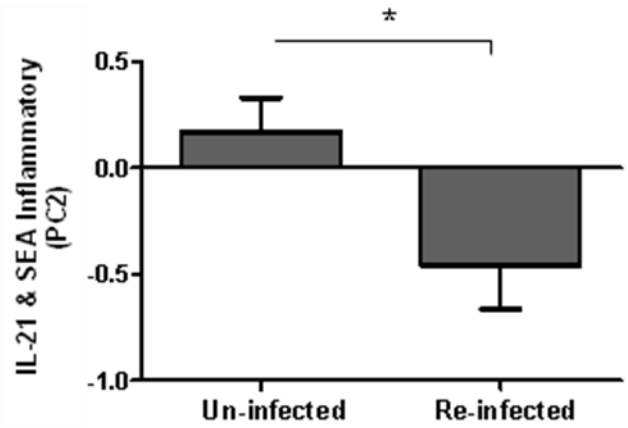


Figure 6.5. Individuals re-infected with *S. haematobium* within 18 months of treatment have lower levels of IL-21 and egg-specific inflammatory cytokines (PC2) than those who remained un-infected. Mean factor scores for adult worm and egg-specific PC2 at 6 weeks post-treatment are plotted by re-infection status within 18 months of treatment. Un-infected (n = 38) and re-infected (n = 15) groups were compared via 2-tailed un-paired t-test, *p<0.05. Error bars: standard error of the mean.

6.4.5 GST-specific cytokine profiles 6 weeks post-treatment and the risk of re-infection

Factor analysis of GST-specific cytokine responses at 6 weeks post-treatment for the 53 individuals with re-infection data extracted 5 PCs (Table 6.9). However, none of these post-treatment GST-specific cytokine profiles were significant predictors of re-infection status within 18 months of treatment (H-L X^2: 9.28, p = 0.320). Results of the analysis are summarised in Table 6.10.

Phenotype	*Cytokine*	*Antigen*	*Principal components*				
			1	***2***	***3***	***4***	***5***
			Inflammatory	Th2/Th1/ Th17	Th2	Innate inflammatory	Th1/ Regulatory
Innate inflammatory	***TNFα***	***GST***	**0.8**	0.3	0.0	-0.2	-0.2
	IL-6	***GST***	**0.9**	0.1	0.1	0.1	0.0
	IL-8	***GST***	0.4	0.0	0.1	**0.8**	0.1
Th1-type	***IFNγ***	***GST***	0.4	0.2	-0.1	-0.1	**0.7**
	IL-2	***GST***	-0.3	**0.6**	-0.2	-0.2	-0.1
	IL-12p70	***GST***	**0.6**	0.1	0.0	**-0.5**	0.0
Th2-type	***IL-4***	***GST***	-0.2	-0.1	**0.6**	-0.3	0.0
	IL-5	***GST***	0.3	0.1	**0.7**	-0.1	0.0
	IL-10	***GST***	-0.3	**0.5**	0.0	-0.1	**0.5**
	IL-13	***GST***	0.0	**0.7**	-0.3	0.1	-0.3
Th17-type	***IL-17A***	***GST***	-0.3	**0.6**	0.4	0.2	-0.3
	IL-21	***GST***	-0.3	0.4	0.3	0.3	0.2
	IL-23p19	***GST***	**0.9**	0.2	-0.1	0.0	0.0
% of total variance			24.8	13.9	10.7	9.5	7.9

Table 6.9. Factor analysis of GST-specific cytokine responses at 6 weeks post-treatment (n = 53). Table shows PCs 1-5 extracted by regression factor analysis and the factor loadings for post-treatment cytokine responses elicited by *S. haematobium* GST. Cytokines with factor loadings ≥0.5 or ≤-0.5 for an extracted PC are highlighted in bold. The cellular immune phenotype with which the cytokines are associated is given for each PC.

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Predictor	*B*	*Wald X^2*	*p*	*Odds Ratio*	*95% CI*
Sex	0.384	0.314	0.575	1.468	0.38 - 5.62
Age	0.036	0.049	0.826	1.037	0.75 - 1.43
Pre-treatment infection intensity	0.002	0.407	0.524	1.002	1.00 - 1.01
GST-specific cytokine profile:					
Inflammatory (PC1)	-0.432	1.101	0.294	0.649	0.29 - 1.46
Th2/Th1/Th17 (PC2)	0.245	0.520	0.471	1.277	0.66 - 2.49
Th2 (PC3)	-0.134	0.166	0.684	0.875	0.46 - 1.67
Innate inflammatory (PC4)	0.360	0.709	0.400	1.433	0.62 - 3.31
Th1/Regulatory (PC5)	-0.544	1.349	0.246	0.580	0.23 - 1.45

Table 6.10. GST-specific cytokine responses at 6 weeks post-treatment do not influence risk of re-infection (n = 53). Results of binary logistic regression analysis of participant re-infection status within 18 months of praziquantel treatment. B – beta coefficient, 95% CI - 95% confidence interval for odds ratio.

6.5 Discussion

Mass treatment programs for schistosomiasis target heterogeneous communities comprising a range of ages and infection intensities (Montresor *et al.* 2002) and this was also the case in Magaya community. Thus, in addition to characterising how schistosome-specific immune responses are changed following treatment (chapter 5), it was also important to investigate whether heterogeneity between participants at the time of treatment influenced the magnitude of this change. Although previous studies have shown that age and pre-treatment infection intensity can influence post-treatment levels of individual antibody and cytokine responses (Roberts *et al.* 1993; Grogan *et al.* 1996b; Mutapi *et al.* 2002; Reimert *et al.* 2006) this is the first study to investigate the effect of these factors on changes in groups of cytokines associated with innate inflammatory, Th1, Th2 and Th17-type responses. Furthermore, an important question raised by observations that schistosome-specific cytokine profiles are changed after treatment is whether these changes affect the risk of subsequent re-infection.

In the first part of this chapter, I investigated the hypothesis that pre-treatment infection levels would influence the magnitude of change in *S. haematobium*-specific whole blood cytokine profiles. This hypothesis stems from the predictions of mathematical models of human schistosome infection, which predict that exposure to parasite antigens is related to parasite density (Woolhouse 1994) and the same has also been assumed of the degree of immunosuppression mediated by live parasites (Mitchell *et al.* 2008). Therefore, since praziquantel treatment leads to parasite death and a release of parasite antigens (Harnett and Kusel 1986; Redman *et al.* 1996), the amount of antigen released after treatment is predicted to be proportionate to the number of parasites present at the time of treatment (i.e. pre-treatment infection intensity). Consistent with this hypothesis I have shown that individuals with high egg counts in their urine prior to treatment had a greater change in a mixed profile of egg-specific cytokines (IFNγ, IL-4, IL-6, IL-12p70 and IL-23) and adult worm-specific IL-4 (PC1) than those who had no detectable schistosome eggs in their urine or low intensity infection.

In contrast to SEA and WWH-specific inflammatory cytokine responses (PC1), the change in IL-21 responses to these antigens (PC4) after treatment was negatively correlated with pre-treatment infection intensity. In chapter 5 I showed that post-treatment IL-21 was higher

than pre-treatment levels in response to all of the *S. haematobium* antigen preparations (CAP, WWH, SEA, and GST) and also in un-stimulated cultures, suggesting that treatment-induced exposure to parasite antigens promotes IL-21 secretion. However the results of this chapter suggest that individuals with high intensity infections had a less pronounced change in IL-21 levels than those harbouring low intensity or un-detectable infections. There are several potential mechanisms that could account for this pattern. Firstly, since participants (particularly young children) with the highest pre-treatment infection intensities also had the highest pre-treatment egg and adult-worm-specific IL-21 responses (chapter 4), the existing high numbers of circulating IL-21+ cells in these participants may mean that IL-21 production cannot increase further, potentially due to the physiological restrictions of the peripheral cell pool (Rocha *et al.* 1989). High and sustained IL-21 secretion before and after treatment may be particularly important in individuals with high intensity helminth infections since observations in experimental *S. mansoni* (Pesce *et al.* 2006) and *Heligmosomoides polygyrus* infections (Fröhlich *et al.* 2007) suggest that IL-21 receptor signalling is required for initiation and maintenance of granulomatous responses to egg antigens, which may persist in host tissues even after clearance of adult parasites..

Alternatively, since IL-21 responses remain similar to pre-treatment levels, but the change in alternative egg-specific effector cytokines (PC1) is the most pronounced in participants with high intensity infection, treatment may render the IL-21 responses to parasite antigens relatively less prominent. As the first study to investigate antigen-specific IL-21 responses in human schistosomiasis it is unclear what the functional relevance of this change might be. However, murine studies suggest that IL-21 may regulate effector immune responses in a variety of contexts, including via induction of alternatively activated macrophages during helminth infection (Pesce *et al.* 2006) and via limiting peripheral T cell expansion (Datta and Sarvetnick 2008). An alternative possibility is that expression of IL-21 may be limited in participants with a large increase in PC1 cytokines, since T-bet, a transcription factor that promotes Th1 differentiation, inhibits IL-21 gene expression by murine T cells (Mehta *et al.* 2005). Clearly, further studies are required to investigate whether the putative roles of IL-21 identified in murine studies apply to human IL-21 and to elucidate the role of this cytokine during schistosome infections.

Of the GST-specific cytokine profiles, PC4 responses were significantly negatively correlated with pre-treatment infection intensity, indicating that the change in adult worm

and egg-specific IL-2 and IL-13 responses was smaller and the change in IL-4 responses was larger in individuals with high intensity infections. The opposite patterns of change in IL-4 and IL-13 provide further support for a context-dependent dissociation between Th2-type effector cytokine responses in human schistosome infections (Scott *et al.* 2000) and, in this case, the change in IL-4 appears to be more closely related to the number of parasites (or exposure of their antigens) than IL-13 responses.

The reciprocal change in GST-specific inflammatory cytokine responses and IL-10 (GST PC1) was also negatively correlated with pre-treatment infection intensity suggesting that individuals with the highest pre-treatment infection intensity had the least change in inflammatory responses and the greatest change in IL-10 responses following treatment. This relationship was only significant in 11-12 year olds, the age group at which mean pre-treatment infection intensities were highest (see chapter 2.3.7). The importance of reciprocal changes in GST-specific inflammatory cytokines and regulatory IL-10 responses may be particularly important in this age group since systemic exposure of GST (Mutapi *et al.* 2005), and GST-specific inflammatory cytokine responses (chapter 5) increase following treatment and may also increase the risk of immunopathology. For example, IL-10 can regulate effector cytokine responses to crude schistosome antigens in whole blood samples collected from schistosome-exposed participants (Grogan *et al.* 1998a; Mutapi *et al.* 2007b). Cross-sectional population studies suggest that pre-treatment titres of schistosome-specific antibody isotypes exhibit reciprocal changes at the age of peak infection intensity that correspond to declining levels of schistosome infection with age and persist into adulthood (Mutapi *et al.* 1997; Mutapi *et al.* 2007b). This pattern has been attributed to exposure to a threshold amount of parasite antigen during cumulative exposure to infection in childhood (Mutapi *et al.* 1998a; Mutapi *et al.* 2008) and is accelerated by praziquantel treatment which increases exposure to schistosome antigens in all age groups (Mutapi *et al.* 1998a; Mutapi *et al.* 2003). Thus increased exposure to GST following treatment may accelerate the development of GST-specific responses in participants with low intensity infections whereas high intensity infections in others may mean that immunological changes dependent on a threshold level of exposure to GST have already occurred before treatment.

This hypothesis is consistent with the observations of a study of *S. haematobium*-specific IgE and IgG4 titres in Gabon, where adults (post-peak infection intensity) were found to have a lower magnitude of change relative to children (pre-peak and peak infection intensity) following treatment (Grogan *et al.* 1996b). Notably the relationship between the change in

GST PC1 cytokines and pre-treatment infection intensity was positive in young children (though not statistically significant) consistent with the effect of treatment on GST-specific cytokine profiles changing with age.

It is interesting that, unlike GST, changes in SEA and WWH-specific cytokine responses after treatment were not related to host age, although this lack of association has also been noted in previous studies of post-treatment immune responses to crude schistosome antigens (Grogan *et al.* 1998b; Mutapi *et al.* 1998b; Joseph *et al.* 2004b). This may be due to the simultaneous and polyclonal activation of innate, naïve and memory cells by the release of antigens from dying worms upon treatment , which would making the relative contributions of antigen-specific memory cells to *in vitro* cytokine responses less distinguishable. Thus, the relationship between the change in SEA and WWH-specific responses and pre-treatment infection intensity may be less clear than for the change in cytokine responses to purified GST. Memory cells are of particular interest in this context since immunological memory is generated by exposure to parasite antigens and these responses may provide the most direct means of assessing how exposure history relates to cellular immunity (Ahmed and Gray 1996). Development of protocols for the isolation and identification of activated cells in cultured whole blood would provide a useful tool to investigate whether effector memory cells to SEA and/or WWH constituents vary with age post-treatment in future studies and to investigate the cell types specifically activated by GST (Maino 1998; Suni *et al.* 1998).

Having characterised the relationship between the change in parasite-specific cytokine responses and pre-treatment infection intensity, I subsequently investigated whether treatment-altered cytokine responses varied between individuals who remained un-infected 18 months after treatment and those that had acquired new infections in this time. Whilst some studies in human helminthiases have focused on how pre-treatment immune responses relate to the risk of re-infection (Mutapi *et al.* 1999; Jackson *et al.* 2004a), I focused on post-treatment cytokine responses since any re-infection will occur in the context of this altered immune environment rather than those observed prior to treatment. Post-treatment SEA-specific inflammatory cytokine responses and adult worm and egg-specific IL-21 (PC2) were associated with a reduced risk of re-infection within 18 months of treatment. This observation is consistent with the long held view that the development of resistance to re-infection is immune-mediated rather than due to age-related changes in water contacts or immune physiology alone (Colley *et al.* 1986; Hagan *et al.* 1991; Roberts *et al.* 1993;

Grogan *et al.* 1998b; Mutapi *et al.* 1999; Caldas *et al.* 2000). It is noteworthy that, although I have classified egg-specific responses associated with a reduced risk of re-infection as 'pro-inflammatory', they reflect a 'mixed' profile of cytokines including Th1-type (IFNγ and IL-12p70) and Th17-type (IL-23) effectors as well as IL-6, which is associated with 'innate inflammatory' responses, but is often up-regulated in conjunction with Th1 effectors (Curfs *et al.* 1997; Oppenheim 2001). Whilst higher schistosome-specific IFNγ has been previously observed in endemically-exposed, but un-infected individuals relative to their infected counterparts (Grogan *et al.* 1998b; El Ridi *et al.* 2001) this is the first study to investigate how IFNγ responses relate to parallel cytokine secretion patterns. In particular, its known ability to promote human macrophage activation (Nathan *et al.* 1984) make it unsurprising that IFNγ co-varies with innate inflammatory cytokines. As discussed above, the absence of mechanistic studies of IL-21 function in humans and particularly during helminth infection make it impossible to conclude whether the positive loading of IL-21 onto a PC with inflammatory cytokines and its association with a reduced risk of re-infection in this context is due to its pro-inflammatory function or co-incidental up-regulated as an immunoregulator or a marker of heightened immune-responses. It is also important to note that, due to the small number of re-infected participants 1 year after treatment (15 of 53 participants included in the re-infection analysis), the statistical power of my observations are limited. Thus, whilst the results of the current chapter have identified a novel association between post-treatment cytokine phenotype and the risk of re-infection, the 'acid test' for these observations will be to investigate whether similar associations can be identified in larger treatment-re-infection cohorts and in other human populations affected by urinary schistosomiasis.

Importantly, since eggs are only produced after an infection has already been established, it is unlikely that immune responses targeting egg antigens alone would lead to a reduced risk of re-infection. What seems more likely is that post-treatment inflammatory cytokine responses to schistosome egg antigens correspond to responses that limit *de novo* infection, migration or maturation of larval parasites or the fitness/fecundity of adult worms. For example, CAP-specific cytokine responses were also increased after treatment (chapter 5) and it is possible that changes in these responses would also be higher in the participants who remained un-infected 18 months after treatment relative to re-infected individuals. Furthermore, SEA contains a range of antigens which cross-react with CAP, WWH and schistosomula antigens (Curwen *et al.* 2004; Jang-Lee *et al.* 2007) and therefore increased

reactivity to egg-stage parasites may also promote anti-larval and anti-adult worm immune responses. For example, whilst induction of 'concomitant immunity' is promoted by adult worms in experimental schistosomiasis (Smithers and Terry 1967) and during chronic filariasis (Day *et al.* 1991a; Day *et al.* 1991b; MacDonald *et al.* 2002), mathematical models predict that schistosome egg antigens may also act as a stimulus for changes in the host immune response associated with resistance to high intensity infection prior to treatment (Woolhouse 1994; Mitchell *et al.* 2008; Mitchell 2010). The observation that effector cytokine responses are higher in un-infected than in re-infected participants is also consistent with the findings of earlier empirical investigations in Egypt, which showed that individuals with low PBMC proliferative responses to *S. mansoni* SEA (and CAP) *in vitro* were more likely to be re-infected after treatment and that this relationship was independent of variation in host age, sex and pre-treatment infection intensity (Colley *et al.* 1986). However, the association between egg-specific cellular proliferative responses to *S. mansoni* antigens and the risk of re-infection remains controversial (Grogan *et al.* 1998b; Caldas *et al.* 2000). For *S. haematobium* the association between immune response and re-infection rates is similarly un-clear (and notably under-studied) and few studies have investigated the association between SEA-specific cytokines and the risk of re-infection. *S. haematobium* SEA-specific antibody responses are known to increase following treatment (Hagan *et al.* 1991; Mutapi *et al.* 1999; van den Biggelaar *et al.* 2002), but previous studies have not identified a protective role for SEA-specific PBMC cytokine responses (Medhat *et al.* 1998). However, the latter study assessed both a restricted subset of cytokines (IFNγ, IL-4, IL-5 and IL-10) and only adolescent boys were included in the cohort (Medhat *et al.* 1998). Thus, the current study provides a more comprehensive analysis of the parasite antigens and cytokine responses that may mediate the development of immune-mediated protection than any previous investigation.

Post-treatment cytokine responses to GST were not related to a reduced risk of re-infection after treatment, despite suggestions that increased exposure to GST following treatment may promote protective immunity (Dupre *et al.* 1999). Since re-infection was defined by the presence of eggs in urine samples it is possible that the GST-specific cytokine responses discussed in this chapter contribute to anti-fecundity effects similar to those seen in animal models (Boulanger *et al.* 1991) and/or that post-treatment antibody responses to GST promoted resistance to re-infection (Grzych *et al.* 1993), although this was not investigated.

6.6 Conclusions

As initially hypothesised, the changes observed in whole blood cytokine responses to SEA, WWH and GST after treatment were influenced by pre-treatment infection intensity. This is consistent with studies suggesting that artificially-induced parasite death may boost immune-responsiveness to parasite antigens (Woolhouse and Hagan 1999). Since variation in S. *haematobium* infection intensity is associated with variation in both the magnitude and phenotype of the whole blood cytokine response, this may also contribute to heterogeneity in the long-term efficacy of praziquantel treatment between individuals included in mass treatment programmes for schistosomiasis.

Of the cytokine profiles identified at 6 weeks post-treatment, SEA-specific inflammatory cytokines (IL-6, TNFα, IFNγ, IL-12p70 and IL-23p19) and egg and adult-worm-specific IL-21 were associated with a reduced risk of re-infection within 18 months of treatment that was independent of age, sex or pre-treatment infection intensity. Thus, the current study suggests that a single dose of praziquantel may promote the development of immune-mediated resistance to *S. haematobium* re-infection in some, but not all treated individuals.

Chapter 7

Systemic and antigen-specific cytokine responses during experimental *Trichuris suis* infection in seasonal allergy sufferers

7.1 Introduction

The prevalence of immune-mediated diseases, including allergies and auto-immunity, is increasing globally (Asher *et al.* 2006). Current immunotherapy for allergies is based on allergen injections (Varney *et al.* 1991; Movérare *et al.* 2000; Wachholz *et al.* 2002), which require repeated doctor visits over several years and pose a significant risk of adverse side-effects (Varney *et al.* 1991). In light of studies showing that host immune responses are modified by nematode and trematode worms (Maizels *et al.* 1993; Maizels and Yazdanbakhsh 2003; Maizels *et al.* 2004; Figueiredo *et al.* 2010), interest has developed in how helminth infection may impact upon the course of immune-mediated diseases (Leonardi-Bee *et al.* 2006; Flohr *et al.* 2009; Feary *et al.* 2011). However, in human populations endemically exposed to parasitic helminths it is difficult to dissect the course of helminth infection from that of allergy and vice versa since there is a lack of helminth naïve controls. An alternative approach is to investigate the immunobiology of experimental helminth infections of allergic individuals in a helminth non-endemic setting, which is the subject of the current chapter.

Recent pilot studies in helminth-naïve humans have shown that ingestion of the ova or larvae of gastrointestinal (GI) nematodes can effectively reduce clinical manifestations of autoimmune disease in the gut (Summers *et al.* 2005a; Summers *et al.* 2005b; Croese *et al.* 2006). However, despite these promising clinical observations, the immunological basis of how helminths alter the course of immune-mediated diseases during short-term infections has not been characterised. In particular, it is unclear how GI helminths may modulate hyper-reactive immune responses to environmental allergens or auto-antigens at sites outside their local gut environment. In fact, a number of studies indicate that ‘helminth therapy’ is

not effective against common allergies in the airways (Feary *et al.* 2009; Bager *et al.* 2010a; Feary *et al.* 2010). Therefore, the aim of this chapter is to investigate how experimental *Trichuris suis* infection affects the systemic cytokine environment and parasite and allergen-specific cytokine responses of a helminth-naïve human cohort with established allergic rhinitis. Furthermore, since the clinical symptoms of the cohort were seasonally-exacerbated by high grass pollen counts, it was possible to investigate how these immune responses changed at different timepoints over the course of the grass pollen season in individuals treated with *T. suis* ova (TSO) relative to un-infected placebo-treated controls. These cytokine analyses are discussed in the context of existing clinical data on the study cohort, who were enrolled in a phase II clinical trial of TSO as an immunotherapy (Bager *et al.* 2010a).

7.2 Hypotheses

- Systemic and antigen-specific cytokine responses differ in individuals treated with *T. suis* ova relative to placebo-treated controls

- Systemic and antigen-specific cytokine responses during the grass pollen season differ to those outside the grass pollen season

- Cytokine responses to *T. suis* excretory/secretory antigens and environmental pollen allergens differ between *T. suis* ova treated and placebo treated groups

7.3 Materials and Methods

7.3.1 Study design

The current study is part of a phase II randomised double-blind, placebo controlled clinical trial of TSO as an immunotherapy for allergic rhinitis (Registration number: R000001298, Trial ID: UMIN000001070). Full details of the study design and ethical permissions are published (Bager *et al.* 2010a) and summarised in chapter 2.4. Briefly, volunteers with pollen allergen-exacerbated allergic rhinitis and no previous exposure to *T. suis* infection were randomly assigned to TSO or placebo treatment groups and received 8 doses of 2500 TSO suspended in sulphate stabilised 0.015moles/L H_2SO_4 or the H_2SO_4 alone (placebo) in double-blinded preparations at 21 day intervals. Blood samples were collected for plasma and PBMC isolation at 3 timepoints; prior to treatment (baseline), during the peak grass pollen season (grass pollen season) and 21 days after the last treatment was administered (end). PBMCs were stimulated in parallel cultures with *T. suis* excretory/secretory products (E/S), grass pollen allergen (g6) and birch pollen allergen (t3). Un-stimulated cultures acted as negative controls for these assays. The design for the study is summarised in Figure 7.1.

For this chapter cytokine responses were quantified in plasma samples and PBMCs isolated from volunteers enrolled in the study to investigate the effect of 3 variables; treatment group, timepoint and antigen-stimulation.

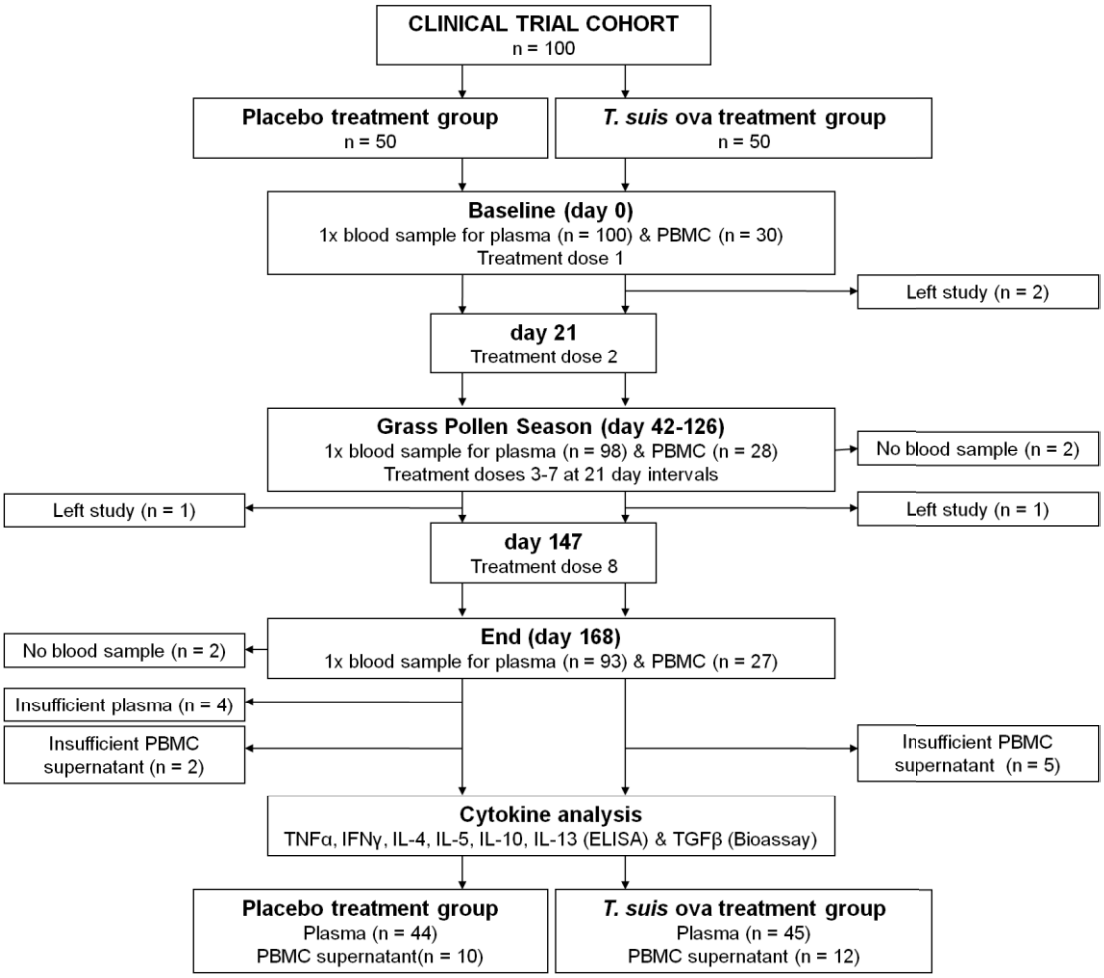

Figure 7.1. Study design and participant record for the assessment of cytokine responses in a clinical trial of *T. suis* ova as an immunotherapy for allergic rhinitis (adapted from (Bager *et al.* 2010a)). Volunteers were randomly assigned to TSO or placebo treatment groups and received 8 treatments at 21 day intervals. PBMCs were isolated from a randomly selected sub-set (n = 15) of each treatment group. Serological samples were collected at 3 timepoints (baseline, grass pollen season and end). Participants who left the study or provided insufficient samples (number or volume) for inclusion in the cytokine analysis are indicated.

7.3.2 Study participants

100 volunteers were initially recruited to the clinical trial and provided a plasma sample prior to ingestion of the first treatment (baseline). Of these, PBMCs were isolated from 30 volunteers.

Participants were included in plasma cytokine analyses according to the following inclusion criteria: 1) completed the 8 dose treatment regime, 2) provided a blood sample at each of the three timepoints (baseline, grass pollen season and end) and 3) provided sufficient sample volume to assay all cytokines (TNFα, IFNγ, IL-4, IL-5, IL-10 and IL-13). For inclusion in the antigen-specific PBMC cytokine cohort individuals were also required to provide a supernatant sample for each of the 3 antigen stimulations: birch pollen allergen (t3), timothy grass pollen allergen (g6) and *T. suis* excretory/secretory product (E/S), and an un-stimulated control at sufficient volume to assay TNFα, IFNγ, IL-4, IL-5, IL-10, IL-13 and TGFβ. 89 individuals met the inclusion criteria for analysis of plasma cytokine responses and, of these, 22 met the inclusion criteria for analysis of PBMC cytokine responses. Details of the number of participants excluded at each stage of the reasons for their exclusion are given in Figure 7.1 and the demographic characteristics of the selected participants are summarised in Table 7.1.

	Plasma cohort		***PBMC supernatant cohort***	
Treatment	***Placebo***	***TSO***	***Placebo***	***TSO***
n	44	45	10	12
Mean age (range)	38.4 (19 - 63)	35.3 (20 - 61)	40.3 (21 - 63)	29.9 (20 - 39)
Gender (m/f)	42/2	43/2	9\1	12/0

Table 7.1. Demographic characteristics of the plasma and PBMC supernatant cytokine study cohorts. Adult volunteers were randomly assigned to either the TSO or placebo treatment groups and a subset of individuals from each group were randomly selected for PBMC isolation and subsequent antigen stimulation. Data in the table relates only to those individuals that met inclusion criteria for cytokine analysis. m – male, f – female

7.3.3 Immunological assays

7.3.3.1 Cytometric bead array (CBA)

CBA was favoured over cytokine ELISA for this aspect of the study since multiple cytokines can be assayed in a smaller volume of plasma/supernatant and available sample volumes were limited. CBA reagents for quantifying IFNγ, TNFα, IL-4, IL-5, IL-10 and IL-13 were purchased from BD Biosciences (CBA Flex Set Assay Cat#: 558269 (IFNγ), 558273 (TNFα), 558272 (IL-4), 558278 (IL-5), 558274 (IL-10) and 558450 (IL-13)). The assay protocol and parameter settings for cytokine quantification were adapted from commercial protocols and the recommendations of a BD Biosciences systems specialist respectively. Plasma cytokine assays were conducted with the help of an undergraduate student, Dana Photiou.

IFNγ, TNFα, IL-4, IL-5, IL-10 and IL-13-specific bead populations were vortexed and pooled in capture bead diluent and 25µl/well added to a 96 well immunoplate (NUNC). Lyophilised recombinant standards for all cytokines were pooled and reconstituted in 4ml assay diluent to give a concentration per cytokine of 2500pg/ml (IL-4, IL-5, IL-10 and IL-13) and 5000pg/ml (IFNγ and TNFα). 9x 25µl/well doubling dilutions were prepared in assay diluent on the plate with a 10^{th} well containing assay diluent alone (blank). 25µl/well of PBMC supernatant samples were added to the remaining wells in duplicate and the plate was incubated at room temperature for 1 hour. Plasma samples were not assayed in duplicate. All incubations were conducted on a plate shaker to prevent aggregation of beads.

PE-conjugated detection antibodies specific for each cytokine were pooled in PE-reagent diluent and 25µl/well added. Plates were incubated for 2 hours at room temperature and protected from light to prevent bleaching of the PE dye. Beads were centrifuged at 200G for 5min and washed twice in wash buffer, before re-suspension in 100µl/well.

Assay plates were read on a BDFACSArray bioanalyser using settings that accounted for optical spill-over of fluorescent dyes and thus maximised assay sensitivity. Quality control assays using SPHERO 8-peak beads (BD Pharmingen, Catalogue#558542) were run prior to

each experiment to ensure that the bioanalyser laser alignment and calibration was identical for each assay.

CBA results were filtered for non-specific fluorescence using the BD FCSFilter program and the same filter gate was used to standardise analysis of all assays. Filtered cytokine data was analysed using BD FCAP software, which quantifies PE fluorescence intensity for each cytokine relative to that of the 10-point recombinant standard curve.

7.3.3.2 TGFβ Bioassay

Heat-activated TGFβ was measured in t3, g6, E/S and un-stimulated PBMC culture supernatants via bioassay using the same supernatant aliquot as that used for CBA cytokine analysis. A bioassay was favoured over CBA or ELISA-based quantification of TGFβ since these methods detect both active and latent forms of TGFβ, the latter of which is biologically inert (Khalil 1999).

The assay uses mink lung epithelial cells (MLEC, clone 32) transfected with a 800bp insert comprising a firefly luciferase reporter gene fused to the 5' end (-799 to +71) of the human plasminogen activator inhibitor-1 (PAI-1) gene (a TGFβ responsive promoter). The insert was introduced as part of a p19LUC-based vector construct containing the neomycin-resistance gene from pMAMneo (Abe *et al.* 1994). Transformed reporter MLEC were provided by Dr. Matthew Taylor, Institute of Immunology and Infection Research, University of Edinburgh and originally generated by Daniel B. Rifkin of the Rifkin Laboratory, Department of Cell Biology, New York University Medical centre.

MLECs were cultured in DMEM with 10% FCS, 100 U/ml penicillin/streptomycin and 100µg/ml L-glutamine and harvested using trypsin/EDTA when confluent. Cells were then diluted to $3.2x10^5$ cells/ml in RPMI with 0.5% mouse sera and 50µl/well added to opaque white fluoroplates (NUNC, Cat#136101). Cells were incubated at 37^{o}C for 3 hours to allow MLEC to adhere. Adherence was assessed in a separate clear-bottomed immunoplate run in parallel with the assay plate.

PBMC supernatant samples were heat activated at 80^{o}C for 5 minutes to release TGFβ from its latent form and 50µl/well added to the MLEC. Recombinant TGFβ standards

(Boehringer) were run in parallel in doubling dilutions from a top concentration of 500pg/ml. RPMI with 0.5% mouse sera was added to duplicate wells to act as a negative control for the assay (blank).

After incubation for 14 hours at 37°C culture plates and Bright-glo luciferase assay substrate (Promega, Cat#E-2620) were equilibrated to room temperature. 100µl/well of substrate was added to each well for 2 minutes to allow cell lysis and plates were then read on a luminometer and TGFβ concentrations interpolated from the standard curve in Excel.

7.3.4 Statistical analyses

Preliminary analysis of supplementary immunological data on eosinophil counts, lymphocyte counts, serum histamine levels, grass pollen and *T. suis* E/S-specific antibody titres and total IgE confirmed that the 2 treatment groups did not differ significantly in any of these parameters prior to treatment in either the plasma or PBMC cytokine cohort (see Appendix 2). These results are consistent with the published analysis of this data including all participants recruited to the original study (Bager *et al.* 2010a).

The effects of timepoint and treatment group on the dynamics of individual plasma and PBMC cytokines were investigated via repeated measures ANOVA. Exploratory analyses indicated that residuals of ANOVA models for both plasma and PBMC cytokines met the assumptions of parametric tests and cytokine concentrations (pg/ml) were subsequently analysed un-transformed. Cytokines were included as dependent variables and compared by timepoint (baseline, peak grass pollen season and end) as a within-subject effect and treatment group (TSO and placebo) as a between-subject effect and the interaction between timepoint and treatment group. The repeated measures design was chosen to account for multiple sampling from the same individual at different timepoints since cytokine responses are expected to be more similar within an individual subject at different timepoints than between different participants (Mutapi and Roddam 2002). Since the difference in variance between each timepoint was unequal, significance levels for univariate tests of the effect of treatment, timepoint and the timepoint-treatment interaction were determined at the Lower-bound epsilon (ε)-adjusted degrees of freedom, which provides a conservative estimate of

significance accounting for un-equal variances (Geisser and Greenhouse 1958; Vasey and Thayer 1987).

Prior to analysis of cytokine responses, antigen-stimulated PBMC supernatant cytokines had levels of cytokine secreted by PBMCs cultured without antigen (un-stimulated controls) subtracted to account for non-specific background cytokine secretion prior to analysis.
PBMC cytokine responses were then compared by timepoint and treatment group using repeated measured ANOVA (described above) conducted separately for each antigen (t3, g6 and E/S).

Temporal variations in the plasma cytokine responses of the 2 treatment groups were further explored via non-metric multidimensional scaling (NMS) conducted using PC-ORD software. The advantage of this approach was that the cytokines contributing to variation between the individuals at all 3 timepoints could be visualised and compared in the context of their co-incident cytokine responses. Prior to analysis concentrations of all cytokines were square-root(x+1)-transformed for each participant to reduce the influence of outlier values on the ordination (Rummel 1970) and to allow participants without detectable levels of one or more cytokines to be included in the analysis (Osborne 2002). The Sorensen (Bray and Curtis) distance between individuals was calculated according to each participant's ranked combination of TNFα, IFNγ, IL-4, IL-5, IL-10 and IL-13 responses at each timepoint. The axes along which participants' cytokine profiles at the 3 different timepoints were plotted reflect cytokine responses that account for the greatest variation within the data set as a whole. Full details of the NMS procedure are given in chapter 2.5.4.

The contribution of different cytokine responses to the variation between individuals in the ordination plot was identified by the Pearson's correlations between the individual cytokine responses and each axis. Only cytokines responses with an $r^2>0.5$ were considered to be adequately reflected by the axis. The amount of variance in participant cytokine responses represented by each axis (i.e. the coefficient of determination (r^2) between the original cytokine profiles and the relative positioning of participants on the ordination plot generated by NMS) is given for each plot. Clusters of participant cytokine responses relative to these axes were identified visually and related to timepoint and treatment group using colour-coded overlays of the ordination plot. The difference between the mean dissimilarities for each treatment group and timepoint were confirmed quantitatively using the multiple

response permutation procedure (MRPP). The test statistic (T), p-value and chance-corrected within-group variation (A, a measure of effect size) are reported for comparisons and full details of how these statistics are calculated is given in chapter 2.5.5.

To address the hypothesis that the difference in cytokine responses between the 2 treatment groups at the 3 study timepoints was influenced by antigen stimulation, NMS was also used to compare PBMC cytokine responses (TNFα, IFNγ, IL-4, IL-5, IL-10, IL-13 and TGFβ) as described above. NMS allowed the patterns of PBMC cytokine responses elicited by *T. suis* E/S, t3 and g6 to be directly compared, which was not possible using repeated measures ANOVA due to differences in the variance of cytokine concentrations between parasite antigen and allergen-stimulated cultures. Each participant was plotted according to their cytokine responses to each antigen (t3, g6 and E/S) at each timepoint (baseline, grass pollen season and end). MRPP was subsequently used to compare cytokine responses between treatment groups, timepoints and the different antigens.

To account for the multiple comparisons made between groups for ANOVA, repeated measures models and MRPP, the sequential Bonferroni-adjustment was used for each set of comparisons (Holm 1979; Rice 1989). Raw p-values are reported for all tests and those significant at the Bonferroni-adjusted significance level are indicated where appropriate.

7.4 Results

7.4.1 Individual plasma cytokine responses by timepoint and treatment group

The immunological impact of TSO was first investigated in plasma samples, which provided an indicator of systemic immune responses at all 3 timepoints (baseline, grass pollen season and end). Levels of IL-5 were significantly higher in *T. suis* infected participants relative to the placebo-treated group. The treatment-dependent increase in plasma IL-5 occurred in the context of a post-treatment increase in eosinophil counts and *T. suis*-specific antibodies (Appendix 2). None of the other plasma cytokines significantly differed between the treatment groups (Table 7.2).

Mean TNFα, IL-4, IL-5 and IL-10 concentrations in both treatment groups were significantly affected by timepoint, suggesting that levels of environmental grass pollen influenced the systemic cytokine response (Table 7.2). Plasma TNFα declined with time and IL-10 was lower at the end of the study than at recruitment. Interestingly, despite their mutual association with Th2-polarised immune responses IL-5 was elevated during the peak grass pollen season relative to the end of the study, but IL-4 levels declined after initial treatment and were lowest during the grass pollen season.

Levels of IL-5 were also significantly affected by the interaction between treatment and timepoint, which confirmed that the elevation of IL-5 in the TSO-treated group above levels in placebo-treated individuals occurred at both post-treatment timepoints (grass pollen and end), but was not evident prior to treatment (baseline).

Mean concentrations of plasma cytokines by timepoint and treatment group are shown in Figure 7.2 and results of repeated measures ANOVA are summarised in Table 7.2.

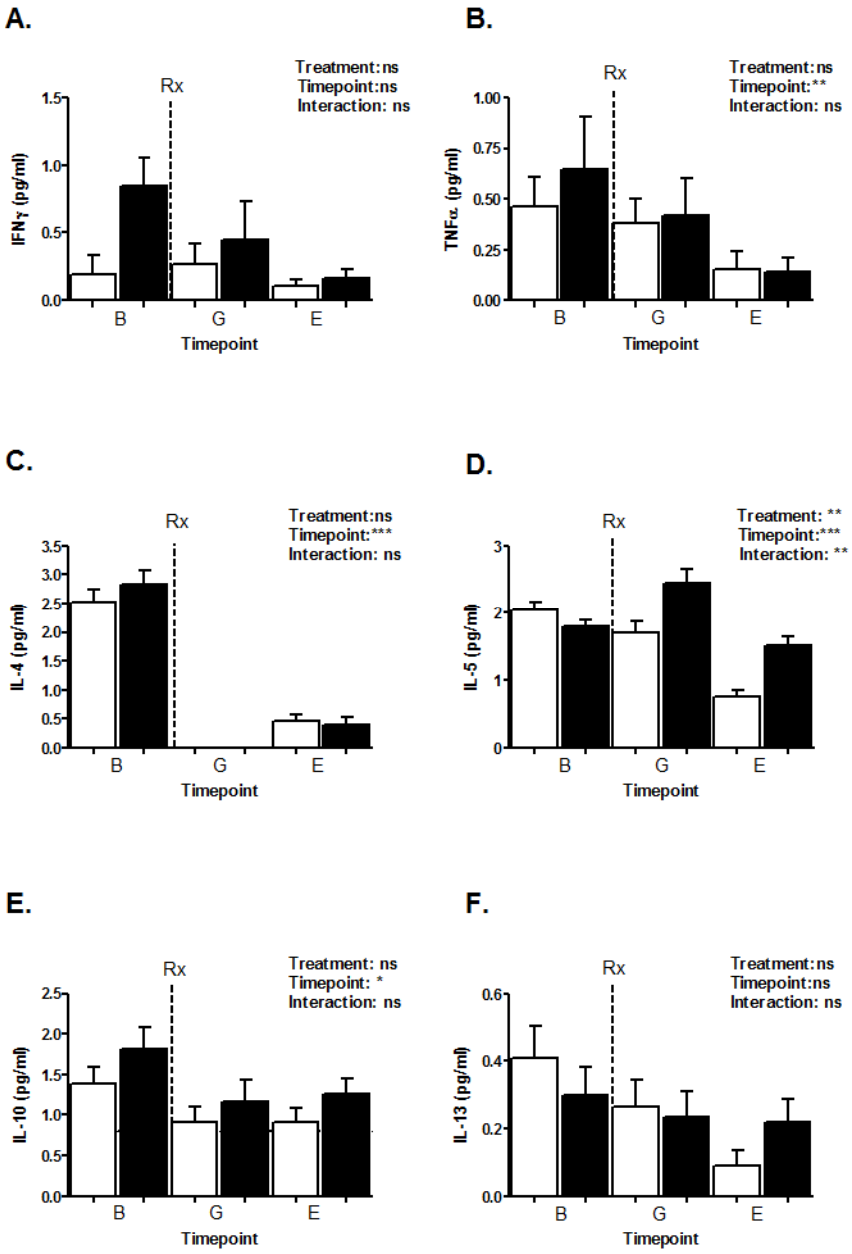

Figure 7.2. Systemic cytokine responses differ according to treatment group, timepoint and the interaction between treatment group and timepoint. Bar charts represent the mean plasma cytokine responses of placebo (white) and TSO (black)-treated groups at baseline (B), during the grass pollen season (G) and at the end of the study (E). Effects of treatment, timepoint and the interaction between treatment and timepoint on cytokine levels assessed by repeated measures ANOVA are indicated for each cytokine. Rx – first dose of treatment administered. Error bars: standard error of the mean. *p<0.05, **p<0.01, ***p <0.001, ns – not significant

Cytokine	*Timepoint*			*Treatment*			*Timepoint* Treatment*	
	F	***p.***	***Post-hoc comparisons***	***F***	***p***	***Post-hoc comparisons***	***F***	***p***
IFNγ	2.634	0.075		0.130	0.720		0.135	0.714
TNFα	8.451	**0.005**	**G > B, E>B**	0.099	0.754		0.475	0.493
IL-4	176.140	**<0.001**	**B > G, B> E, E>G**	0.287	0.593		0.618	0.434
IL-5	32.963	**<0.001**	**B > E, G > E**	9.539	**0.003**	***T. suis*>Placebo**	11.247	**0.001**
IL-10	9.961	**0.002**	**B > G, B > E**	2.869	0.094		0.495	0.484
IL-13	3.465	0.066		0.006	0.936		1.920	0.169

Table 7.2. Systemic cytokine responses differ according to treatment group, timepoint and the interaction between treatment group and timepoint. Results of repeated measures ANOVA of plasma cytokine responses are summarised and significant differences are highlighted in bold. Results significant after sequential Bonferroni correction for multiple comparisons are shaded grey. Treatment*Treatment – interaction between treatment group and timepoint. B –baseline, G –grass pollen season, E – end. Degrees of freedom (Lower-bound ε-adjusted): 1, 87

7.4.2 Parasite and allergen-specific PBMC cytokine responses

Having identified an association between TSO and temporal variations in grass pollen and the systemic cytokine environment, I investigated whether this was also the case for *T. suis*-specific and allergen-specific PBMC cytokine responses in a subset of the cohort from whom PBMC were isolated (TSO group: n = 12, placebo-treated group: n = 10). Assaying the cytokine responses of *T. suis* E/S (Figure 7.3), g6 (Figure 7.4) and t3 (Figure 7.5)-stimulated PBMCs allowed the influence of different stimuli on peripheral cellular immune responses to be assessed. Mean concentrations of *T. suis* E/S, g6 and t3-specific PBMC cytokines are plotted by treatment group and timepoint in Figures 7.3, 7.4 and 7.5 respectively. Results of repeated measures ANOVA are summarised for *T. suis* E/S-specific cytokine responses in Table 7.3, t3-specific responses in Table 7.4 and g6-specific responses in Table 7.5.

Consistent with initial hypotheses, mean concentrations of *T. suis* E/S-specific PBMC cytokines differed according to treatment group. Concentrations of all Th2-type cytokines (IL-4 (Figure 7.3C), IL-5 (Figure 7.3D) and IL-13 (Figure 7.3F)) and IL-10 (Figure 7.3E) were significantly higher in TSO-treated participants relative to placebo controls. The latter is consistent with induction of a Th2/regulatory cytokine response by helminth secreted antigens.

PBMC cytokine responses also differed according to timepoint. Levels of IFNγ (Figure 7.3A) and IL-10 (Figure 7.3E) peaked during the grass pollen season. The latter also appeared to be the case for mean concentrations of *T. suis*-E/S-specific IL-4, IL-5, IL-10 and IL-13; however this trend was not statistically significant.

Of the allergen-specific PBMC cytokine responses, only levels of TGFβ to birch pollen significantly differed between the 2 treatment groups (Figure 7.4G). Mean t3-specific TGFβ concentrations were higher in *T. suis*-treated group relative to placebo controls, but were not significantly affected by the interaction between the effects of treatment group and timepoint. None of the birch or grass pollen allergen-specific cytokine responses differed significantly between timepoints.

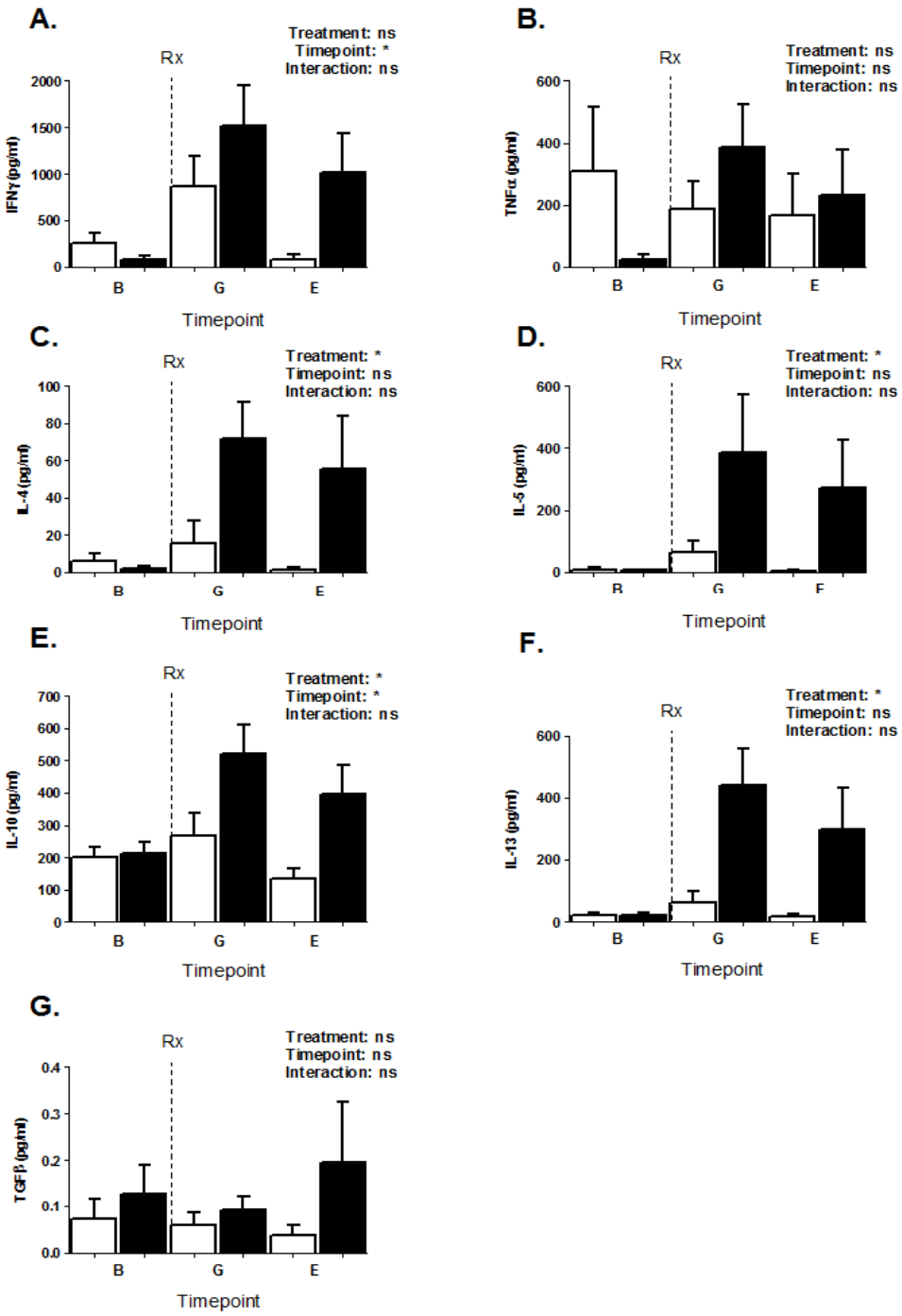

Figure 7.3. Levels of *T. suis* E/S-specific PBMC cytokines are affected by treatment group and timepoint. Bar charts represent the mean *T. suis* E/S-specific cytokine responses of placebo (white) and TSO (black)-treated groups at baseline (B), during the grass pollen season (G) and at the end of the study (E). Effects of treatment, timepoint and the interaction between treatment and timepoint on cytokine levels assessed by repeated measures ANOVA are indicated for each cytokine. Rx – first dose of treatment administered. Error bars: standard error of the mean. *$p<0.05$, ns – not significant

Cytokine	*Timepoint*			*Treatment*			*Timepoint*Treatment*	
	F	***p***	***Post-hoc comparisons***	***F***	***P***	***Post-hoc comparisons***	***F***	***p***
IFNγ	6.723	**0.017**	**G > B, G> E**	1.617	0.218		2.126	0.160
TNFα	0.247	0.625		0.160	0.693		3.415	0.079
IL-4	2.975	0.100		5.396	**0.031**	***T. suis*>Placebo**	2.109	0.162
IL-5	1.844	0.190		4.635	**0.044**	***T. suis*>Placebo**	1.352	0.259
IL-10	7.972	**0.010**	**G > B, G > E**	4.374	**0.049**	***T. suis*>Placebo**	2.663	0.118
IL-13	4.205	0.054		7.955	**0.011**	***T. suis*>Placebo**	3.162	0.091
TGFβ	0.239	0.630		1.661	0.212		0.614	0.443

Table 7.3. *T. suis* E/S-specific PBMC IFNγ and IL-10 responses change over time and Th2-type cytokine responses differ according to treatment group. Results of repeated measures ANOVA of PBMC cytokine concentrations elicited by *T. suis* E/S stimulation *in vitro* are summarised and significant differences are highlighted in bold. None of the results were significant after sequential Bonferroni correction for multiple comparisons. B –

baseline, G – grass pollen season, E – end. Degrees of freedom (Lower-bound ε-adjusted): 1, 20

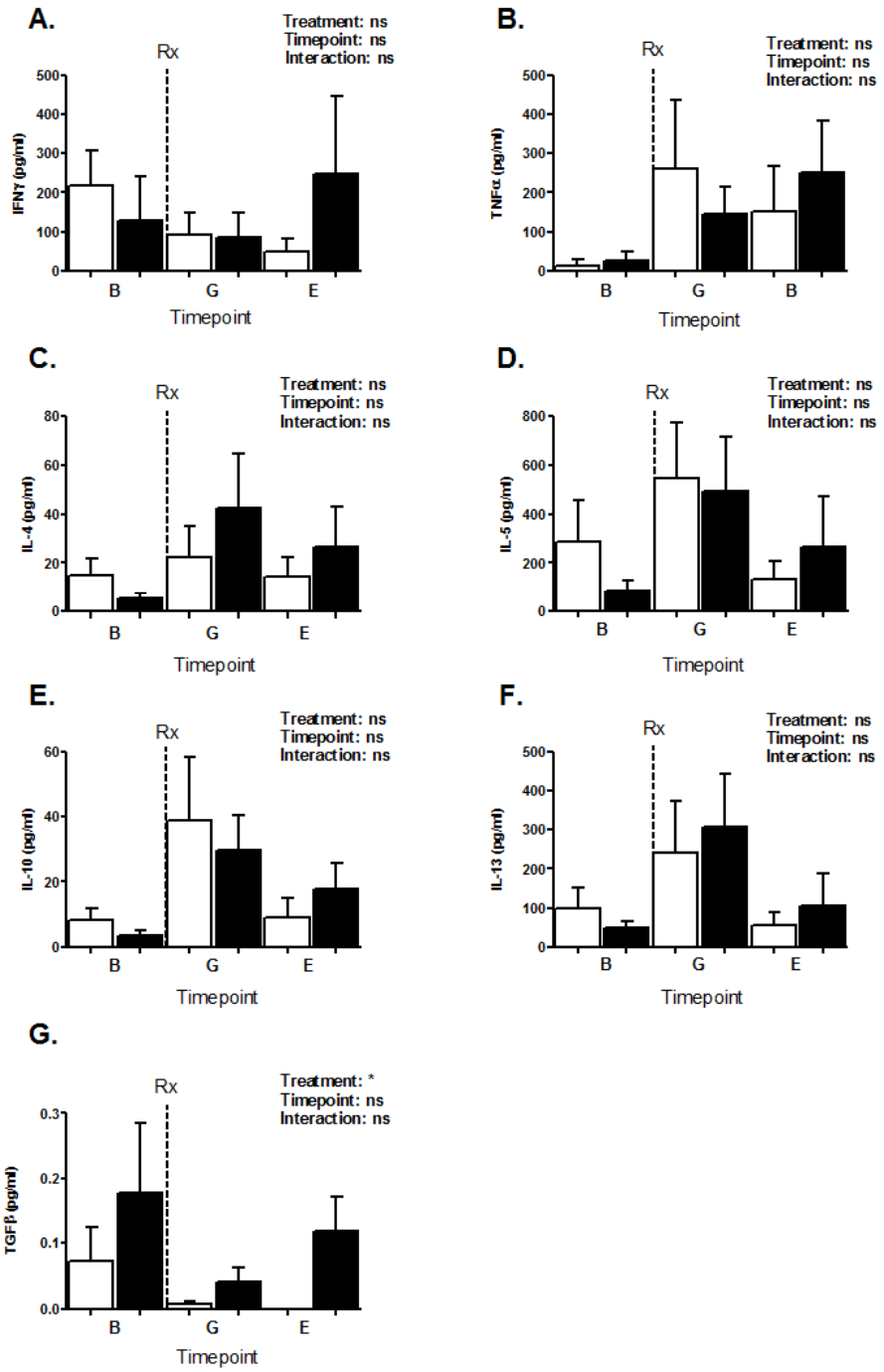

Figure 7.4. Birch pollen-specific PBMC TGFβ responses are higher in the *T. suis* ova-treated group than in the placebo-treated group. Bar charts represent the mean t3-specific cytokine responses of placebo (white) and TSO (black)-treated groups at baseline (B), during the grass pollen season (G) and at the end of the study (E). Effects of treatment, timepoint and the interaction between treatment and timepoint on cytokine levels assessed by repeated measures ANOVA are indicated for each cytokine. Rx – first dose of treatment administered. Error bars: standard error of the mean. *$p<0.05$, ns – not significant

Cytokine	*Timepoint*			*Treatment*			*Timepoint*Treatment*	
	F	***P***	***Post-hoc comparisons***	***F***	***P***	***Post-hoc Comparisons***	***F***	***p***
IFNγ	0.558	0.577		0.020	0.890		1.108	0.340
TNFα	1.461	0.241		0.091	0.766		0.208	0.653
IL-4	1.731	0.203		0.102	0.753		0.695	0.414
IL-5	2.953	0.101		0.075	0.786		0.894	0.356
IL-10	3.128	0.092		0.037	0.849		0.339	0.567
IL-13	3.822	0.065		0.001	0.974		0.337	0.568
TGFβ	0.847	0.368		8.241	**0.009**	***T. suis*>Placebo**	0.499	0.488

Table 7.4. Birch pollen-specific PBMC TGFβ responses differ according to treatment group. Results of repeated measures ANOVA of PBMC cytokine concentrations elicited by t3 stimulation *in vitro* are summarised and significant differences are highlighted in bold. None of the results were significant after sequential Bonferroni correction for multiple comparisons. Degrees of freedom (Lower-bound ε-adjusted): 1, 20

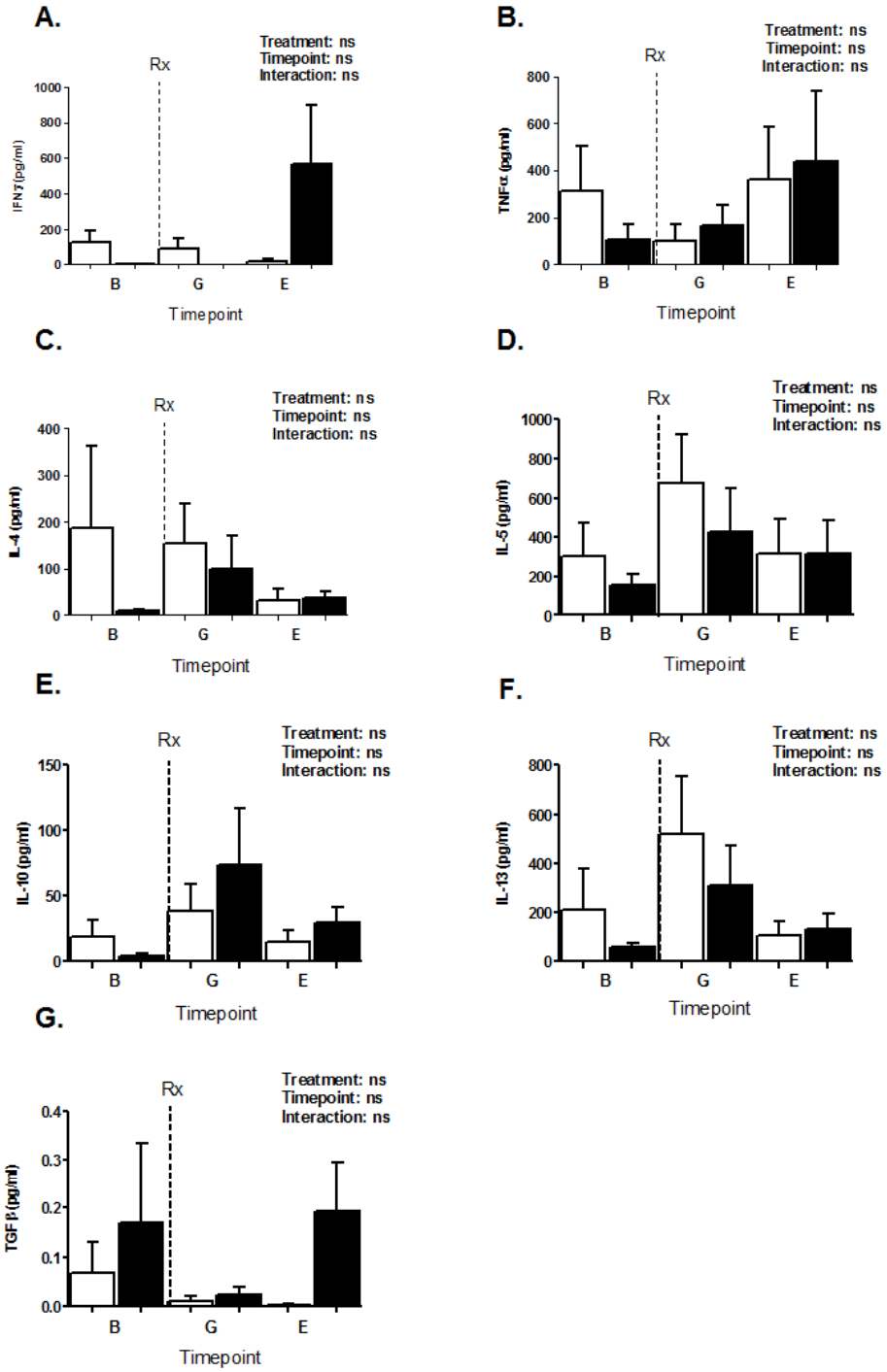

Figure 7.5. Grass pollen allergen-specific PBMC cytokine responses are not significantly affected by *T. suis* ova treatment or timepoint. Bar charts represent the mean g6-specific cytokine responses of placebo (white) and TSO (black)-treated groups at baseline (B), during the grass pollen season (G) and at the end of the study (E). Effects of treatment, timepoint and the interaction between treatment and timepoint on cytokine levels assessed by repeated measures ANOVA are indicated for each cytokine. Rx – first dose of treatment administered. Error bars: standard error of the mean. ns – not significant

Cytokine	*Timepoint*			*Treatment*			*Timepoint*Treatment*	
	F	***p***	***Post-hoc comparisons***	***F***	***P***	***Post-hoc comparisons***	***F***	***P***
IFNγ	1.394	0.252		0.405	0.531		3.503	0.076
TNFα	0.747	0.398		0.336	0.569		0.455	0.508
IL-4	0.817	0.377		1.107	0.305		0.892	0.356
IL-5	2.635	0.120		0.259	0.616		0.546	0.469
IL-10	2.342	0.142		0.056	0.816		0.468	0.502
IL-13	3.559	0.074		0.580	0.455		0.702	0.412
TGFβ	0.460	0.505		3.545	0.074		0.592	0.450

Table 7.5. Grass pollen-specific PBMC cytokine responses are not affected by timepoint or treatment group. Results of repeated measures ANOVA of PBMC cytokine concentrations elicited by g6 stimulation *in vitro* are summarised. None of the factors were found to significantly affect PBMC cytokine concentrations. Degrees of freedom (Lower-bound ε-adjusted): 1, 20

7.4.3 Temporal variations in systemic cytokine responses due to *T. suis* ova treatment

Since individual cytokine responses occur in the context of multiple cell types and co-incident cytokines I sought to investigate whether mean dissimilarities between participant cytokine profiles (i.e. the difference between the combined IFNγ, TNFα, IL-4, IL-5, IL-10 and IL-13 responses of each participant and those of other participants at all timepoints) also differed according to treatment group and timepoint. NMS identified 2 main axes, corresponding to the major sources of variation between participant cytokine profiles. Axis 2, which had a strong positive correlation with IL-4 responses (Table 7.6), accounted for the greatest proportion of variation between participant cytokine profiles (Figure 7.6). Axis 1 accounted for a lower proportion of variation (Figure 7.6) and was positively correlated with IL-10 responses (Table 7.6). Despite treatment and timepoint-specific patterns of plasma IL-5 in isolation (see above), IL-5 was not strongly correlated (Pearson's $r>0.5$) with either axis. Participant cytokine profiles are plotted relative to each other in NMS ordination plots (Figure 7.6) and identified by timepoint (Figure 7.6A) and treatment group (Figure 7.6B) in colour-coded overlays. The final 2-dimensional NMS of plasma cytokine responses had a final stress of 19.1 and instability of 0.002 after 500 iterations.

Participant cytokine profiles formed distinct clusters along the NMS axes according to timepoint and mean dissimilarities in IL-4 (axis 2) were higher at baseline than at either of the post-treatment timepoints (Figure 7.6A), consistent with the individual cytokine analysis described above. MRPP confirmed that cytokine profiles differed according to timepoint (T: -56.267, $p<0.001$, A: 0.152) and pair-wise comparisons indicated that cytokine profiles differed significantly between participants at all timepoints (baseline vs. grass pollen season T: -53.88, $p<0.001$, A: 0.159, baseline vs. end T:-44.83, $p<0.001$, A: 0.139, grass pollen season vs. end T: -14.68, $p<0.001$, A: 0.039).

There was considerable overlap between the cytokine profiles of the 2 treatment groups at all timepoints; however mean dissimilarities in IL-10 responses (axis 1) were higher in TSO-treated individuals than placebo-treated controls (Figure 7.6B). MRPP confirmed that the cytokine responses significantly differed according to treatment group (T:-3.662, $p = 0.007$),

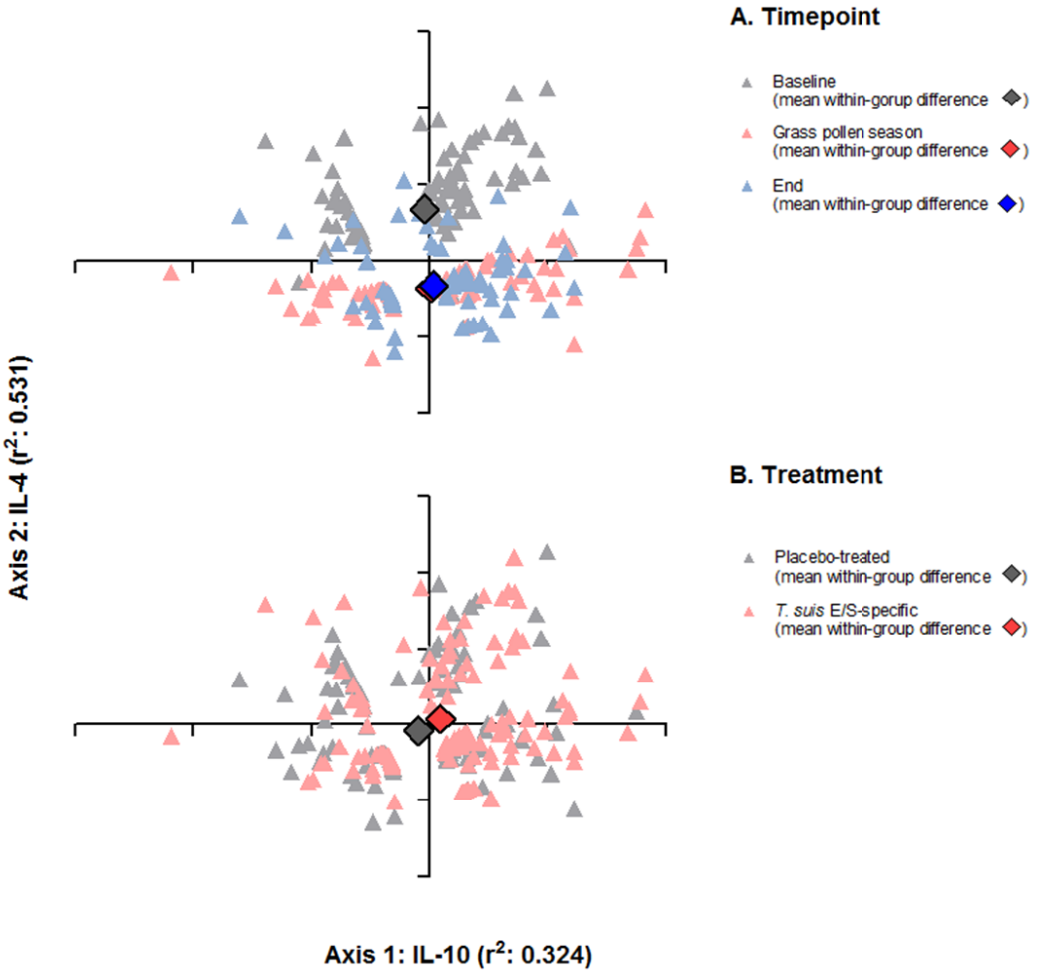

Figure 7.6. Plasma cytokine responses are influenced by timepoint and treatment group (n = 89). Participants are plotted according to relative dissimilarities in their plasma cytokine responses and groups are identified by overlays of the ordination plot according to timepoint (A) and treatment group (B). Cytokines correlating with each axis with a Pearson's r^2>0.5 and the proportion of total variance accounted for by each axis (r^2) are indicated. Mean within-group difference - mean dissimilarities between participant cytokine responses in each timepoint (A) or treatment group (B).

Plasma cytokines	*Axis 1*		*Axis 2*	
	Pearson's r	r^2	*Pearson's r*	r^2
IFNγ	0.2	0.0	0.3	0.1
TNFα	0.1	0.0	0.4	0.2
IL-4	-0.1	0.0	**0.9**	**0.7**
IL-5	0.0	0.0	0.4	0.2
IL-10	**0.8**	**0.6**	0.5	0.2
IL-13	0.2	0.0	0.3	0.1
Proportion of variance:	0.324		0.531	

Table 7.6. Temporal variation in plasma cytokine profiles relate to changes in IL-10 and IL-4. The correlation coefficient (Pearson's r) and coefficient of determination (r^2) for each cytokine relative to the 2 dimensional axes identified by NMS are given. Cytokine responses with $r^2 > 0.5$ are highlighted in bold and shaded grey. Proportion of variance refers to the r^2 value of each ordination axis relative to the original square-root(x+1)-transformed cytokine data.

although the variation within both groups meant that the effect size of this difference was low (A: 0.007).

7.4.4 Variation in PBMC cytokine profiles due to timepoint, treatment group and antigen stimulation

When participants' combined PBMC cytokine profiles was assessed by NMS, variation between participants was attributable to 2 main types of cytokine responses: 1) a combination of IFNγ and IL-10 responses, which correlated positively with axis 1 and 2) a combination of IL-5 and IL-13 responses, which correlated negatively with axis 2 (Table 7.7).

Ordination plots indicated that PBMC cytokine profiles formed clusters according to antigen stimulus (Figure 7.7). *T. suis* E/S-specific PBMC responses were higher on axis 1 and lower for axis 2 than those elicited by the 2 allergens (Figure 7.7A), which corresponds to higher levels of IFNγ, IL-5, IL-10 and IL-13 in *T. suis* E/S-stimulated cultures and the induction of a more mixed Th1/Th2/regulatory-type immune response by *T. suis* antigens relative to allergen-specific cytokine profiles. MRPP confirmed that mean dissimilarities between participant cytokine profiles differed according to antigen with significant differences between *T. suis* E/S and g6 and E/S and t3, but not between the 2 allergens (Table 7.8).

Participant PBMC cytokine profiles to all antigens overlapped between timepoints (Figure 7.7B); however timepoint was still a significant contributor to variation between antigen-specific cytokine profiles. Mean dissimilarities of participant responses during the grass pollen season were distinct along both axes relative to those at baseline and the end of the study (Figure 7.7B). These differences were confirmed by pair-wise comparisons using MRPP (Table 7.8). Cytokine profiles did not significantly differ between the baseline and end timepoint (Table 7.8).

PBMC cytokine responses of the 2 treatment groups (Figure 7.7C) were similar for all antigens and at all timepoints as can be seen from overlap between the TSO and placebo-treated participants in Figure 7.7C. MRPP confirmed that dissimilarities in PBMC cytokine responses did not significantly differ according to TSO treatment (Table 7.8).

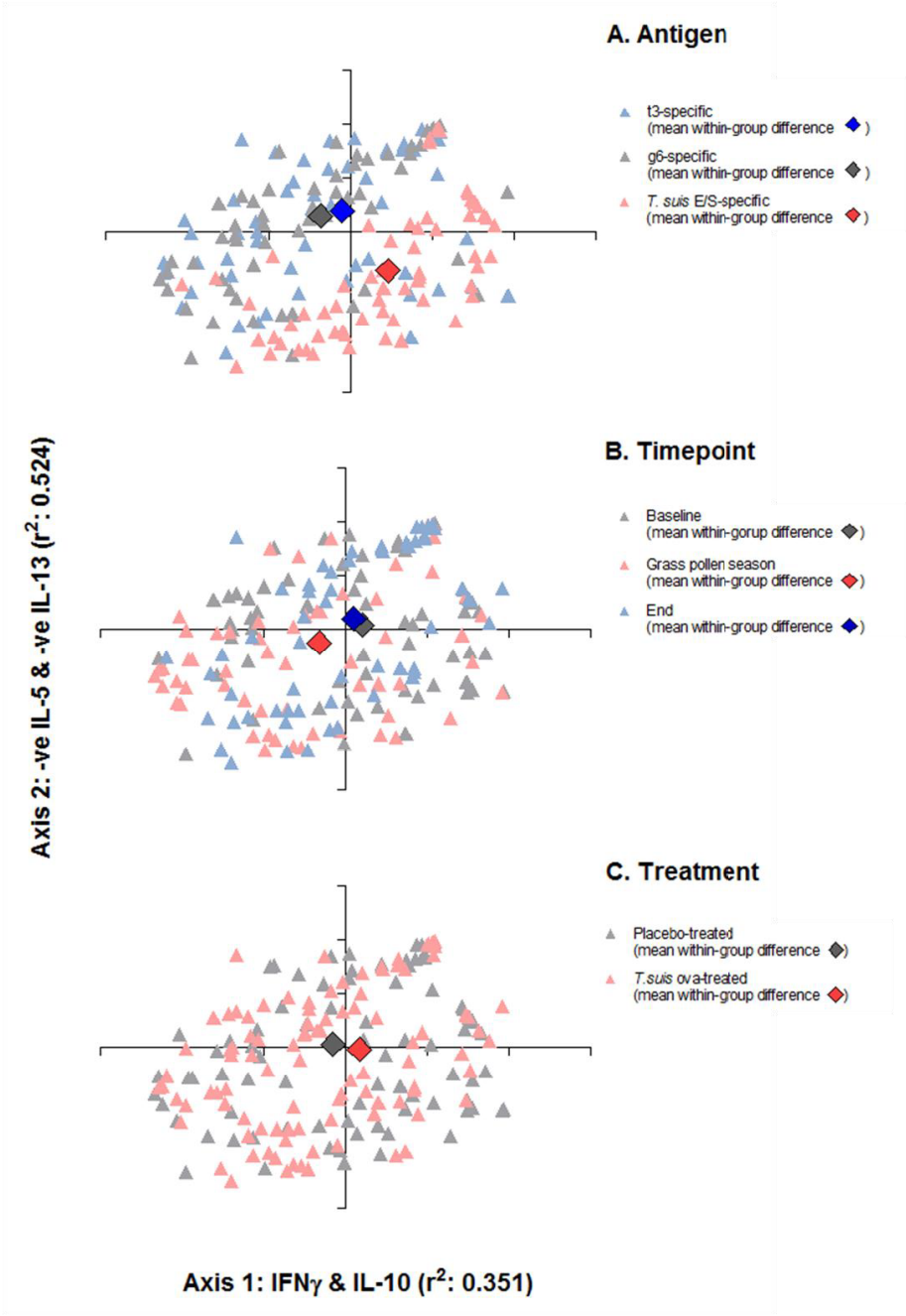

Figure 7.7 PBMC cytokine responses are influenced by the type of antigen stimulation and timepoint (n = 22). Participants are plotted according to relative dissimilarities in their PBMC cytokine responses and groups are identified by overlays of the ordination plot according to antigen stimulation (A), timepoint (B) and treatment group (C). Cytokines correlating with each axis with a Pearson's r^2>0.5 and the proportion of total variance accounted for by each axis (r^2) are indicated. Mean within-group difference – mean dissimilarities between participant cytokine responses for each antigen (A), timepoint (B) and treatment group (C)

PBMC cytokines	*Axis 1*		*Axis 2*	
	Pearson's r	r^2	*Pearson's r*	r^2
IFNγ	**0.8**	**0.6**	-0.4	0.1
TNFα	0.4	0.2	-0.6	0.3
IL-4	0.2	0.1	-0.7	0.4
IL-5	0.1	0.0	**-0.8**	**0.7**
IL-10	**0.7**	**0.5**	-0.4	0.1
IL-13	0.3	0.1	**-0.8**	**0.7**
TGFß	0.1	0.0	-0.1	0.0
Proportion of variance:	0.351		0.524	

Table 7.7. Temporal patterns of parasite and allergen-specific PBMC cytokines profiles relate to differences in IFNγ and IL-10 (Axis 1) and IL-5 and IL-13 (Axis 2). The correlation coefficient (Pearson's r) and coefficient of determination (r^2) for each cytokine relative to the 2 dimensional axes identified by NMS of PBMC cytokine responses are given. Cytokine responses with $r^2 > 0.5$ are highlighted in bold and shaded grey. Proportion of variance refers to the r^2 value of each ordination axis relative to the original square-root(x+1)-transformed cytokine data.

Variable	*Pair-wise comparisons*	*T statistic*	*p*	*A*
	t3 vs. G6	0.159	0.435	-0.001
Antigen	***t3 vs. T. suis E/S***	-25.92	**<0.001**	0.095
	g6 vs. T. suis E/S	-25.99	**<0.001**	0.095
	Baseline vs. Grass pollen season	-4.49	**0.003**	0.016
Timepoint	***Baseline vs. End***	-1.07	0.131	0.004
	Grass pollen season vs. End	-2.72	**0.024**	0.024
Treatment	***T. suis vs. Placebo***	-0.964	0.145	0.002

Table 7.8. PBMC antigen-specific cytokine responses differ according to the type of antigen and timepoint. Table gives results of MRPP comparison of mean Sorensen (Bray and Curtis) dissimilarities between each antigen (t3 – birch pollen allergen, g6 – grass pollen allergen and E/S – *T. suis* excretory/secretory product), timepoint (B – baseline, G – grass pollen season and E - end) and treatment group (TSO and placebo-treated). Significant differences ($p<0.05$) are highlighted in bold and those significant after Bonferroni correction for multiple comparisons are shaded grey. A – chance-corrected within-group agreement

7.5 Discussion

Parasitic helminth infection can modulate the host immune response to parasite and non-parasite antigens and may contribute to the lower prevalence of allergic disease in helminth-endemic areas relative to areas where helminths are non-endemic or have been eradicated (Yazdanbakhsh *et al.* 2002; Fumagalli *et al.* 2009). What is less clear is whether experimental helminth infections may exert similar effects in helminth-naïve individuals already sensitised to allergens. The current study, which assayed cytokine responses of participants enrolled in a clinical trial of TSO therapy for allergic rhinitis (Bager *et al.* 2010a), allowed both the cytokine responses to experimental helminth infection and the effect of infection on allergen-specific responses to be investigated. Since all participants were allergic to grass pollen allergen, cytokine responses were expected to be influenced both by changes in grass pollen counts over time and, for infected cases, the development of an anti-parasite response. The results of this study show for the first time that ingestion of repeated doses of TSO alters the systemic and PBMC cytokine environment of helminth naïve human hosts relative to placebo-treated controls. Although previous studies of cytokine responses to single low-dose experimental hookworm infections in allergic rhinitis sufferers lead to transient changes in PBMC cytokine responses (Blount *et al.* 2009), this is the first study to demonstrate that repeated doses of *T. suis* ova induce more sustained alterations to spontaneous and *T. suis*-specific cytokine responses. In addition to the general observation that the host cytokine environment is altered by experimental helminth infection, the results of the study highlight 3 major characteristics of the cytokine response to *T. suis*: 1) experimental infection and *T. suis* antigens elicit a Th2-polarised response, 2) Th2-cytokines exhibit distinct dynamics according to the treatment received by each participant and the timepoint at which responses were assayed and 3) *T. suis* infection does not significantly influence allergen-specific cytokine responses in the periphery. The latter observation is consistent with initial predictions that PBMC cytokine responses elicited over the course of the study would vary according to antigen stimulation.

The study showed that repeated doses of TSO result in the maintenance of systemic IL-5 responses, which declined during the grass pollen season in the placebo-treated group. The difference between the 2 treatment groups was most pronounced at the end of the study at which point the *T. suis* treated cohort had received the maximum dose of parasite ova. Since *T. suis* E/S-specific PBMC IL-5, but not allergen-specific IL-5, was elevated in the infected

group it also seems likely that temporal changes in plasma IL-5 reflect the course of *T. suis* infection rather than changes in environmental grass pollen counts over the course of the study. Elevated plasma IL-5 responses coincided with higher *T. suis*-specific antibody titres and eosinophil counts (refer to Appendix 2 and published data (Bager *et al.* 2010a)) in the infected group. *T. suis* E/S also up-regulated Th2-type PBMC cytokine (IL-4, IL-5, IL-10 and IL-13) responses, as has been seen in numerous *in vitro* studies of cytokine responses to helminth antigens (e.g. schistosome eggs (Schramm *et al.* 2003; Everts *et al.* 2009) and somatic hookworm antigens (Wright and Bickle 2005)). Although temporal changes in these cytokines indicative of an increase in Th2 responses after initial infection were observed, these were not statistically significant, which may reflect both the small size of the PBMC cohort (n = 22) and variability of their cytokine responses. In addition to Th2-type cytokines, *T. suis* E/S elicited significantly elevated levels of PBMC IFNγ in both the infected and un-infected groups during the grass pollen season, indicating that the E/S preparation may also contain Th1/inflammatory stimuli. Similarly soluble egg antigens (SEA) of schistosome parasites, which are known to be potent Th2-inducers, contain Toll-like receptor (TLR) ligands capable of inducing innate inflammatory cytokine secretion (van der Kleij *et al.* 2002a; van der Kleij *et al.* 2004).

In contrast to plasma IL-5, plasma IFNγ, TNFα, IL-4, IL-10 and IL-13 responses were not significantly affected by treatment. Similar to the IL-5 responses of the placebo-treated group but in contrast to those of the TSO-treated group, TNFα, IL-4 and IL-10 levels declined relative to baseline levels in both treatment groups. The dissociation between Th2-type cytokine responses, has also been previously observed in studies of natural helminth infections in endemically-exposed populations (Grogan *et al.* 1996a; Sartono *et al.* 1997; Scott *et al.* 2000) and in murine models of chronic immune hyper-reactivity in the airways (Leigh *et al.* 2004). Given the increased proliferation of IL-4 producing cells in *T. suis*-infected pigs (Steenhard *et al.* 2007) and the association between IL-4 and chronic GI helminth infections in humans (Cooper *et al.* 2000), it was particularly surprising that plasma IL-4 levels did not differ between the *T. suis*-infected group and placebo controls. However, during porcine *T. suis* infections IL-4 production appears more pronounced in the ileo-caecal lymph nodes than in peripheral blood samples and, if these changes in IL-4 occur in our human cohort, they may be too subtle to detect in peripheral plasma samples (Steenhard *et al.* 2007). Perhaps more surprising is that levels of plasma IL-4 declined in both treatment groups during the grass pollen season, during which peripheral IL-4 responses might be

expected to peak (Benson *et al.* 1997). This was not due to an absence of clinical allergy since over 80% of the cohort reported moderate/severe allergic symptoms during the course of the study (Bager *et al.* 2010a). However, the decline in plasma IL-4 during the grass pollen season relative to baseline observed here is corroborated by observations in human hay-fever sufferers in whom IL-4 levels were reduced during the grass pollen season relative to baseline levels despite exacerbated allergy and elevated eosinophil markers (Jones *et al.* 2000). Thus, whilst IL-4 appears to be a key mediator of local immune responses in allergen-sensitised tissues (e.g. in human nasal fluid (Benson *et al.* 1997; Scavuzzo *et al.* 2003)), it is possible that IL-4+ cells are recruited to and sequestered in the airway mucosa when pollen counts lead to exacerbated allergy. In the case of T cells the latter is supported by observations in human allergic rhinitis sufferers in whom spontaneous and pollen allergen-specific PBMC IL-4 production was lower during the grass pollen season (Jepsen *et al.* 1998), but inconsistent with observations in experimental human hookworm infection of allergic rhinitis sufferers where circulating T cell numbers and IL-4+ T cells were unaffected by infection (Blount *et al.* 2009). For eosinophils, an IL-4-dependent increase in eosinophil chemoattractants and receptors by endothelial cells (Foster 1996; Schnyder *et al.* 1996) would be expected to limit the number of IL-4+ eosinophils and other eosinophil-derived cytokines detectable in the periphery. Murine models also suggest that cytokine responses can be highly localised at the site of allergen sensitisation and un-detectable in adjacent tissues (Denburg *et al.* 1990; Saito *et al.* 2001). In light of these studies, IL-4 may not be the best peripheral correlate of human allergic rhinitis, as has been suggested by others (Jones *et al.* 2000). Collection of nasal lavage fluids or biopsy samples to complement plasma and PBMC cytokine measurements and/or assaying alternative markers of allergy (e.g. eosinophil cationic protein (Jones *et al.* 2000)) would provide further evidence to support/refute this hypothesis in future studies.

Of the allergen-specific PBMC cytokine responses assayed, only birch pollen-specific TGFβ significantly differed significantly in the TSO-treated group relative to placebo controls. Mean grass pollen allergen and *T. suis* E/S-specific PBMC TGFβ responses were also higher in the TSO-treated group, although these patterns were not statistically significant. However, since TGFβ was not significantly affected by timepoint it is unclear whether the marginally higher mean concentrations in the *T. suis*-infected group at baseline or the effect of subsequent treatment was responsible for this difference at the post-treatment timepoints. There was also considerable variation in PBMC cytokine responses between individuals,

particularly in response to grass pollen allergens, which may have been due to the range of grass pollen sensitivities within the cohort identified at recruitment (Bager *et al.* 2010a). Since sensitivity was detected by skin-prick test (Bager *et al.* 2010a), it was not possible to quantify this variation in the current study. This could be addressed in future studies if PBMC samples were collected from a larger cohort or clinical atopy was scored for all participants and accounted for in statistical comparisons.

With respect to the original aim of the clinical trial (i.e. to alleviate allergic rhinitis symptoms), induction of IL-10 secretion by *T. suis* antigens *in vitro*, an IL-10-polarised plasma cytokine profile in infected individuals and the treatment-associated increase in birch pollen allergen-specific TGFβ all indicate the potential of TSO therapy to induce immunoregulatory cytokines. However, no clinical improvement in the TSO-treated group relative to the placebo-treated group was recorded over the course of the clinical trial (Bager *et al.* 2010a). One reason for this may be that the induction of 'regulatory responses' during infection occurred in the context of heightened effector cytokine responses (IFNγ, IL-4, IL-5, IL-10 and IL-13) to *T. suis* E/S and systemic IL-5, which may have limited the therapeutic efficacy of treatment. Consistent with this hypothesis IL-10 and IFNγ responses (axis 1), which are associated with regulation of excessive Th2 polarisation and allergen-induced inflammatory pathology (Borish *et al.* 1996; Comoy *et al.* 1998; Wosinska-Becler *et al.* 2004), were less pronounced during the grass pollen season relative to the Th2-effector cytokines IL-5 and IL-13 (axis 2), as might be expected during exacerbated allergic airway responses. These observations are consistent with immunological studies of *T. suis* infection in pigs, which show that effector Th2 responses develop readily within weeks of infection (Kringel *et al.* 2006; Kringel and Roepstorff 2006; Steenhard *et al.* 2007). Thus, since *T. suis* infection is spontaneously cleared by infected humans (Summers *et al.* 2005c), significantly more doses of TSO may be required to induce the immunosuppressive environment associated with chronic helminth infections (Yazdanbakhsh *et al.* 2002; Jackson *et al.* 2009).

It is also possible that elevated IL-5, eosinophil counts, histamine levels and parasite-specific IgE in *T. suis* infected participants (see Appendix 2 and published study (Bager *et al.* 2010a)) may have exacerbated rather than ameliorated airway hyper-reactivity (Foster *et al.* 1996; Wosinska-Becler *et al.* 2004). It is noteworthy that during the clinical trial adverse GI symptoms (including discomfort and diarrhoea) were more prevalent in the TSO-treated group relative to the placebo controls (Bager *et al.* 2010b). Of the plasma cohort, 46.7% of

the placebo-treated cases and 73.3% of the *T. suis*-treated cases reported gastrointestinal discomfort or diarrhoea between initial treatment and the end of the study. In the PBMC supernatant cohort 50% of the placebo-treated cases and 91.7% of the TSO-treated cases reported GI symptoms post-treatment. Although the trial by Bager and colleagues is the first to report adverse side-effects of TSO treatment, the co-incidence of eosinophilia and adverse GI symptoms has been consistently noted in trials of experimental hookworm infection (Croese *et al.* 2006; Daveson *et al.* 2009a; Feary *et al.* 2009). Since the current study is reliant on peripheral blood samples it is unclear whether these responses were specifically elevated in the GI tract where the parasites reside and/or in the airways where allergic symptoms manifest.

A recent commentary on the clinical results of the trial (Bager *et al.* 2010a) has suggested that provision of treatment for GI side-effects in the *T. suis*-treated group may have abrogated development of allergy-regulating responses (Summers *et al.* 2010). Since some participants received only 2 doses of TSO prior to the peak grass pollen season, it is also possible that the dose of parasites was insufficient to induce therapeutic immunoregulatory responses achieved during trials of TSO therapy for Crohn's disease (Hepworth *et al.* 2010; Summers *et al.* 2010). In light of this discussion, a more important consideration may be that diarrhoeal episodes, particularly between baseline and the grass pollen season (Bager *et al.* 2010a), may have cleared or reduced establishment of adult parasites in the gut independent of the number of TSO doses or treatment of side-effects. Whilst presence of parasite-specific antibodies indicates recent exposure to infection (Bager *et al.* 2010a; Bager *et al.* in press), without an indicator of infection intensity (e.g. faecal egg counts) it is unclear whether this was the case in the current study. Unfortunately, the small number of participants and high prevalence of GI symptoms meant that it was not possible to account for the incidence of side effects in cytokine analyses or exclude cases on this basis.

7.6 Conclusions

This study has shown that experimental *T. suis* infection alters both the plasma and antigen-specific cytokine environment of the human host. In particular, infected individuals have higher levels of plasma IL-5 and on stimulation with *T. suis* E/S their PBMCs secrete more IL-4, IL-5 and IL-10 than placebo-treated controls. *T. suis* infection may also enhance TGFβ responses to birch pollen allergens.

Cytokine responses varied temporally, reflecting immunological changes due to the duration of exposure to *T. suis* and variation in environmental grass pollen counts and, in the case of plasma IL-5, treatment related changes were timepoint-dependent. However, the cytokine environment elicited by *T. suis* infection did not alter grass-pollen allergen-specific responses, which may underlie the lack of clinical efficacy reported during the original trial of TSO therapy for allergic rhinitis (Bager *et al.* 2010a)

Thus, the current study provides preliminary evidence that a small number of doses of TSO induce potentially relevant immunological changes in a *T. suis*-naïve allergic cohort. These changes were observed within 2 months of enrolment in the study. Thus it is possible that with an increased duration of infection and adjusted dose of TSO the proposed immunotherapeutic applications of *T. suis* infection in allergic rhinitis may be realised and the observed side-effects avoided (Bager *et al.* 2010a).

Chapter 8

General Discussion

In this thesis I have investigated whether our increased understanding of murine cellular immunology, particularly identification of novel effectors and cross-regulation between different cellular phenotypes (Diaz and Allen 2007; Jenkins and Allen 2010), can improve our understanding of the human immune phenotype during parasitic helminth infection. In individuals naturally exposed to *S. haematobium* infection I have shown that cercariae elicit a more pro-inflammatory cytokine profile than those elicited by adult worms and eggs (chapter 3). *S. haematobium*-specific cytokine profiles also differ according to participant age and the changing relationship between regulatory and Th17-polarised responses and infection intensity with age are consistent with these responses contributing to epidemiological patterns of infection (chapter 4). In chapters 5 I have shown that anti-helminthic treatment not only alters parasite-specific cytokine responses, but leads to a specific increase in pro-inflammatory responses particularly to egg antigens. Treatment-induced changes in the schistosome-specific cytokine profile were dependent on pre-treatment infection intensity and were also associated with a reduced risk of re-infection 18 month after treatment (chapter 6), suggesting that both the effects of treatment and its implications for resistance to infection are dependent on parasite exposure. During experimental *T. suis* infections in a helminth-naïve cohort I have shown that both systemic and parasite-specific cytokine profiles become Th2-polarised relative to un-infected controls (chapter 7), but infection does not significantly alter cytokine responses to allergens (chapter 7).

In this chapter I will discuss how these findings relate to one another and the initial aims of the study (chapter 2.2) and how they extend existing literature on the immunobiology of human helminth infections. I will also highlight key questions raised by this thesis and propose how these questions might be addressed by future studies.

8.1 Why characterise the 'cytokine environment' in human helminth infection?

Cytokines responses reflect both the type and magnitude of cellular immune responses to helminth infections (Pearce *et al.* 1991; Montenegro *et al.* 1999a; Diaz and Allen 2007; Mutapi *et al.* 2007b; de Morais *et al.* 2008; Milner *et al.* 2010) and thus provide an indicator of how parasites may prime different cellular effector phenotypes during infection. Throughout this thesis I have attempted to extend the observations of previous studies suggesting that it is the balance between different immune responses rather than the dynamics of individual molecules that determines anti-parasite and anti-pathology immunity (e.g. IgE and IgG4 (Hagan *et al.* 1991; Demeure *et al.* 1993), IgA and IgG1 (Mutapi *et al.* 1997), TNFα and IL-10 (Wamachi *et al.* 2004), IL-5 and IL-10 (Mutapi *et al.* 2007b) and Teff:Treg (Nausch *et al.* 2011)). Whilst the latter has been inferred from a limited number of antibody or cytokine responses in previous studies, this is the first study to simultaneously assay Th1, Th2, Th17 and innate inflammatory-type responses in human schistosomiasis (chapters 3-6). It is also the first study showing that schistosome-specific cytokines associated with the Th17 lineage vary in response to different parasite antigens (chapter 3), relate to epidemiological patterns of infection (chapter 4), are altered by treatment (chapter 5) and contribute to resistance to re-infection post-treatment (chapter 6).

These observations strongly support further investigation of Th17 responses during human schistosomiasis, particularly in light of murine studies suggesting that they may mediate egg-induced granulomatous pathology (Rutitzky *et al.* 2008; Shainheit *et al.* 2008; Rutitzky *et al.* 2009) and the association of these responses with exacerbation of chronic inflammatory diseases in humans (Juszczak and Glabinski 2009; Hammerich *et al.* 2011) and lymphedema in filarial helminth infections (Babu *et al.* 2009).

In addition to advocating an extension to the current cytokine panels used in human studies, the results of this thesis suggest that the way in which immunological data is analysed can be improved. Although factor analysis and non-metric multidimensional scaling (NMS) have been used to analyse immunological data in only a small number of studies to-date (Turner *et al.* 2003; Wilson *et al.* 2008; Milner *et al.* 2010; Groer and Beckstead 2011; Imai *et al.* 2011), they are routinely used for analysis of large and heterogeneous ecological data-sets where, like cytokine data, multiple variables are quantified on a range of scales, rarely meet parametric assumptions, and exhibit temporal and inter-dependent non-linear variations that are un-detectable using alternative statistical methods (Moore-Kucera and Dick 2008;

Walker 2008; Smith *et al.* 2009; Turcotte *et al.* 2009). Comparing the results of the individual cytokine analyses to the results of the factor analysis of the same responses conducted in chapter 3 clearly shows that the dynamics of individual cytokines can be accurately and more concisely reflected by a single factor analysis. Furthermore, NMS ordination plots provide an easily interpretable 2-dimensional representation of these complex patterns and their reliability can be readily assessed using diagnostic criteria (e.g. stress values, stability criteria, Monte-Carlo randomisation tests (McCune and Grace 2002)) as seen in chapters 5 and 7.

8.2 Do cytokine responses to schistosome cercariae, adult worm and egg antigens inherently differ?

A common feature of many parasitic helminths is a complex phasic life-cycle, progression through which has been shown to alter the host immune response in experimental models (Grzych *et al.* 1991; Pearce *et al.* 1991). A number of immunoepidemiological studies in human schistosomiasis also suggest that, even when hosts are simultaneously exposed to multiple life-cycle stages during chronic infection, the cytokine responses to eggs and adult worms may differ (El Ridi *et al.* 1997; Joseph *et al.* 2004a; Joseph *et al.* 2004b). It has long been proposed that resistance to schistosome infection may develop via reduced accrual of new infections over years of exposure, allowing patent infections to plateau and then decline as adult worms die, but cercariae are prevented from reaching maturity (see chapter 1.6). However, for this to be the case *in vivo* hosts must inherently respond to cercariae differently from how they respond to adult worms and eggs. Thus one of the initial aims of this thesis (chapter 2.2) was to directly compare the cytokine profiles elicited these life-cycle stages.

Proteomic comparisons of crude homogenate antigens of schistosome cercariae, adult worms and eggs provide a precedent for antigenic differences between the 3 life-cycle stages (Curwen *et al.* 2004). Furthermore, whilst studies in the 1970s, 80s and 90s have compared the magnitude of immune responses to CAP, WWH and SEA, including PBMC proliferation (Todd *et al.* 1979; Gazzinelli *et al.* 1983; Colley *et al.* 1986; Contigli *et al.* 1999), *in vitro* granuloma formation (Contigli *et al.* 1999), comparison of isotype-specific serum antibody titres (Butterworth *et al.* 1985; Viana *et al.* 1995) and isolation and culture of T cell clones (Contigli *et al.* 1999), none have investigated whether these patterns are due to phenotypic differences in the immune environment. Thus my observations in chapter 3 that cercarial

antigens elicit a more pro-inflammatory profile of cytokine response than adult worm and egg-stage antigens provides evidence for distinct polarisation of immune responses at different stages of the schistosome life-cycle. For example, pro-inflammatory cytokine responses appear to dominate the early immune response to cercariae as they invade the skin during murine infections (Hogg *et al.* 2003a; Jenkins *et al.* 2005a). In contrast, polarisation of the host immune response by egg antigens, which elicited higher levels of Th2 and Th17-type cytokines than cercariae in the current study (chapter 3), may reflect the putative role of Th2 and Th17-type cytokines in granuloma formation around eggs trapped in host tissues (Chiaramonte *et al.* 1999; Rutitzky *et al.* 2008; Shainheit *et al.* 2008). Thus these results represent an important step forward in our understanding of how exposure to different parasite life-cycle stages may influence human immune responses and the types of effector cytokines that may predominate in different tissues and at different stages of infection.

Inherent differences in the cytokine profiles elicited by cercariae, adult worms and eggs were also evident 6 weeks after treatment (chapter 5), when chronic infection had been removed. But contrary to pre-treatment responses, and despite an increase in pro-inflammatory cytokine responses to cercariae, schistosome eggs elicited the most pro-inflammatory cytokine profile. Although early studies in murine *S. mansoni* have led to the supposition that immature parasites are relatively un-affected by treatment when compared with adult worms (Andrews 1985; Sabah *et al.* 1986; Shaw 1990), my observations suggest that adult worm death and the associated release of adult worm antigens are not the sole effect of treatment and raise the possibility that immature infective and transmission-stage schistosomes may also be targeted by praziquantel as has been observed in laboratory animals (Flisser *et al.* 1989; Giboda and Smith 1994; Liang *et al.* 2001) and *in vitro* (Harnett and Kusel 1986). There are 2 main hypotheses that may account for the increase in cytokine and antibody responses to parasite antigens after treatment and both would be consistent with increased responsiveness to egg and cercariae. Firstly, treatment may release a burst of parasite antigens by damaging their tegument and revealing somatic antigens to immune recognition. Increased exposure to egg antigens after treatment, evident from the elevated egg-specific antibody titres (Mutapi *et al.* 1998b), increased levels of egg antigens in urine (Nibbeling *et al.* 1998) and protracted excretion of eggs (Tchuem Tchuente *et al.* 2004) identified after treatment-induced clearance of infection, suggest that schistosome egg antigens may be particularly relevant stimuli for post-treatment immune responses. Alternatively, parasite death may alleviate immunoregulatory mechanisms that limit immune responses during chronic infection. The latter may be particularly evident for egg and

cercariae-specific effector cytokine responses due to their immunopathogenic potential in the skin (Appleton 1984), liver (Dunne and Pearce 1999; Wilson *et al.* 2008) and urogenital tract (Wamachi *et al.* 2004).

Differential polarisation of cellular immune responses is an area of active interest for immunological studies. For example, murine models routinely use schistosome eggs as Th2-polarising agents to investigate type 2 immune processes (Grzych *et al.* 1991; Wynn *et al.* 1994; Fallon and Dunne 1999; Okano *et al.* 2001; van de Vijver *et al.* 2006) and there are already several constituent antigens of crude schistosome egg antigens known to polarise the cytokine responses of human cells *in vitro* (van der Kleij *et al.* 2002b; Schramm *et al.* 2003; Everts *et al.* 2009; Steinfelder *et al.* 2009). Thus, the current study could be extended to identify the functional roles of stage-specific cytokine phenotypes and may also facilitate 'fine-tuning' of future vaccines (e.g. via modification of proteins or combining different antigens in a single vaccine) to promote immune responses associated with anti-parasite immunity.

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8.3 Do the schistosome-specific cytokine responses contribute to the development of anti-parasite immunity?

In this thesis I have investigated how peripheral cytokine responses relate to 2 scenarios proposed to generate immune-mediated resistance to schistosome infection: 1) evidence from epidemiological patterns of infection that resistance to high intensity infections develops gradually with age/exposure to infection (Woolhouse 1998) (chapter 4) and 2) removal of infection by treatment alters exposure to parasite antigens and promotes resistance to subsequent re-infection (chapter 6) (Woolhouse and Hagan 1999; Mutapi *et al.* 2003; Mutapi *et al.* 2005). The former hypothesis is supported by cross-sectional studies showing that reciprocal changes in schistosome-specific cytokine (Mutapi *et al.* 2007b) and antibody (Mutapi *et al.* 1997) responses correspond to the age-dependent decline in infection intensity and I therefore sought to extend these studies. The latter hypothesis is more controversial since although anti-helminthic treatment has been previously shown to elicit beneficial changes in the parasite-specific immune response (Mutapi *et al.* 1998a; Mutapi *et al.* 2003; Mutapi *et al.* 2005; Watanabe *et al.* 2007) longitudinal treatment-re-infection studies suggest that it has only short-term effects on infection intensity and associated

immunopathology even after repeated doses (van den Biggelaar *et al.* 2002; Guidi *et al.* 2010).

8.3.1 What cytokine profiles are associated with natural development of resistance to schistosomiasis?

To investigate whether pre-treatment schistosome-specific cytokine responses are related to the development of a protective immune phenotype it was first necessary to define which participants could be considered as 'resistant' to infection. Previous studies have used 2 main approaches; whilst some have relied on participant infection status or intensity (Viana *et al.* 1995; Corrêa-Oliveira *et al.* 1998; Al-Sherbiny *et al.* 2003), I have favoured an age-structured analysis as advocated by numerous previous empirical and theoretical studies in human helminthiases (Hagan *et al.* 1991; Woolhouse 1992, 1994; Grogan *et al.* 1996b; Turner *et al.* 2003; Quinnell *et al.* 2004b; Mutapi *et al.* 2008). This approach is favourable since it reflects consistently observed population-level patterns of schistosome infection (reviewed by Woolhouse, 1998). Furthermore, the aggregation of infection in certain age groups and by gender means that defining resistance solely by their current infection levels leads to a biased distribution of ages and sexes between groups (e.g. Al-Sherbiny *et al.*, 2003). Both age and sex are also known to influence infection and immunity (Fulford *et al.* 1992; Webster *et al.* 1997b; Fulford *et al.* 1998) and were also influential factors for current infection levels in Magaya community (chapter 4).

It is not practically or ethically feasible to follow a single individual's levels of infection from initial exposure without providing treatment, therefore an age-structured approach to characterising resistance is based on the assumptions that; a) cross-sectional patterns of schistosome infection in the community reflect patterns of infection experienced throughout life and b) exposure to infection is relatively constant for permanent residents of an area where transmission is stable and endemic. Thus an important aspect of this study was to characterise potential sources of variation in cytokine responses (e.g. co-infection, residential and treatment history and water contact behaviour) within the cohort in order to validate these limitations/assumptions prior to immunological analyses. In particular, extensive water contact surveys conducted as part of previous studies in endemic areas indicate that, as would be expected, the risk of infection is dependent on how often individuals are exposed to 'water contact' sites (i.e. sites of parasite transmission) (Chandiwana and Woolhouse

1991; Woolhouse *et al.* 1991; Rudge *et al.* 2008). However, in this study age-dependent variations in water contact behaviour alone were not responsible for the distribution of infection by age (chapter 4). The latter meant that, cross-sectional patterns of infection in Magaya community could be more reliably related to exposure history (Woolhouse 1992, 1994, 1998) since young children (5-10 years), who were considered as being likely to accumulate increasing levels of infection, and older children and adults (13+ years), who were considered as having developed resistance to high intensity infections, had equivalent mean infection levels and contact with sites of parasite transmission (chapter 4.4.1). 11-12 year olds were considered to be in a transitory stage whereby they had the highest intensity infections of the 3 age groups, but according to cross-sectional patterns of infection in the population as a whole, these levels were predicted to decline with age. Inclusion of all 3 age groups was considered an important extension to studies comparing the immune responses of 'children' and 'adults' alone (e.g. Grogan *et al*. 1996; Nausch *et al*. 2011) since children in the 11-12 age group represent a distinct stage of immune development evident from their greater range of infection intensities (0 - 639 eggs/10ml urine) and distinct parasite-specific cytokine responses relative to 5-10 and 13+ year olds both before and after treatment (chapters 4 and 6).

Although numerous previous studies support an age-dependent change in immune responses or a relationship between immune responses and infection after accounting for the effects of age (chapter 1.6), I investigated both patterns since infection and immunity are inter-dependent and both are influenced by participant age. The results of these analyses are consistent with an exposure-dependent shift in schistosome-specific cytokine responses contributing to the development of immune-mediated resistance to infection. This assertion is supported firstly by the observation that young children mount a more regulatory/Th17-polarised immune response to infection than 11-12 or 13+ year olds (chapter 4). However, as initially suggested for parasite-specific antibodies (Woolhouse 1992; Mutapi *et al.* 1999), a difference between cytokine profiles with age is insufficient evidence for the contribution of these responses to immune-mediated resistance to infection since an immune response will correlate positively with infection intensity if it is stimulated by parasites and their antigens but not involved in clearance of infection (i.e. 'marker of infection'), if it is directly involved in clearance of infection (i.e. 'protective') but also stimulated by parasite antigens in a population with high infection prevalence or if it is co-produced with/activated by/regulated by another immune response that is a 'marker of infection' or 'protective'. Importantly, I have also shown that the relationship between schistosome infection intensity and

schistosome-specific regulatory/Th17-polarised responses changed from a significant positive correlation in 5-10 year olds to no correlation in 11-12 and 13+ year olds (chapter 4). This pattern is consistent with observations that immunological changes with age may correspond with the development of resistance to helminth infections (Quinnell *et al.* 1995; Turner *et al.* 2003; Mutapi *et al.* 2006; Nausch *et al.* 2011), but extends these previous studies by inclusion of a broader range of cytokines, antigen-specificities and age groups within a single population that had not received prior treatment. In contrast to previous studies assaying only a small number of cytokines, I could exclude the possibility that these patterns simply reflected those of other (un-measured) cytokine responses. However, it will be important in future studies to characterise what other soluble molecules, cellular effectors and functional effects can be attributed to a regulatory/Th17-polarised immune response to schistosome antigens.

8.3.2 Does anti-helminthic treatment promote resistance to re-infection?

Another important indicator of anti-parasite immunity is whether endemically-exposed individuals are resistant to re-infection post-treatment in the context of continued exposure to infective cercariae. In order for treatment to promote immune-mediated resistance to re-infection it is first necessary to demonstrate that treatment changes the immune response to parasite antigens. A number of studies provide a precedent for a treatment-dependent change in the immune profile of schistosome-exposed cohorts (Grogan *et al.* 1996b; Mutapi *et al.* 1998a; Mutapi *et al.* 2002) and treatment also affects different cytokines differently (Medhat *et al.* 1998; van den Biggelaar *et al.* 2002; Joseph *et al.* 2004b; Reimert *et al.* 2006; Mduluza *et al.* 2009). However, studies focusing only on the responses to adult worm antigens (Medhat *et al.* 1998; van den Biggelaar *et al.* 2002) or systemic responses (Reimert *et al.* 2006) provide a skewed impression of how treatment influences the host's cytokine profile. The results of chapter 5 show that treatment not only affects different cytokine responses differently, but these effects correspond to a shift in the overall cytokine polarisation of an individual. Furthermore, consistent with a boost in exposure to parasite antigens (Mutapi *et al.* 2005), but in contrast to the assumption that these changes are mainly in response to adult worm antigens (Roberts *et al.* 1993; Joseph *et al.* 2004b), I identified a marked increase in pro-inflammatory cytokine responses to CAP, SEA and GST, but not WWH, relative to pre-treatment responses. Since resistance to infection appears to develop from a decline in parasite-specific regulatory/Th17-type cytokine responses with age/exposure prior to

treatment (chapter 4), the increased exposure to parasite antigens upon treatment might be expected to accelerate their reduction allowing effector responses to predominate. Consistent with this hypothesis, the observed increase in pro-inflammatory cytokine responses occurred in the absence of an increase in parasite-specific IL-10 (with the exception of cercariae-specific IL-10) (chapter 5), which is known to reduce levels of effector cytokines in human whole blood cultures (Grogan *et al.* 1998a; Mutapi *et al.* 2007b). Notably, although parasite-specific and spontaneous IL-21 responses increased in response to all antigens and in un-stimulated cultures after treatment, unlike pre-treatment patterns these responses were not associated with post-treatment IL-10. Collectively these results suggest that treatment induces a switch from a relatively hypo-responsive cytokine profile to adult worm and egg antigens, which do not elicit significantly different profiles prior to treatment (chapter 3), to a dramatic increase in egg-specific effector responses (chapter 5). Furthermore, whilst pro-inflammatory cytokines were not associated with resistance to infection before treatment (chapter 4), egg-specific pro-inflammatory cytokines and adult-worm and egg-specific IL-21 were higher in individuals who remained infection-free than in their re-infected counterparts (chapter 6). Thus, in addition to reducing infection prevalence and intensity (Tchuem Tchuente *et al.* 2004; Midzi *et al.* 2008a), praziquantel treatment may also lead to a generalised increase in immune responsiveness that may boost anti-parasite immunity. Similar associations with a reduced risk of re-infection have also been noted for schistosome-specific antibody titres (Hagan *et al.* 1991; Mutapi *et al.* 1999; Caldas *et al.* 2000) and PBMC proliferative responses (Colley *et al.* 1986). Since both the boost in cytokine responses to crude parasite antigens post-treatment (chapter 5) and the association between post-treatment cytokine profiles and resistance to re-infection (chapter 6) were independent of host age, my findings also suggest that treatment may alter the relationship between age, exposure history and immune responses observed prior to treatment (chapter 4). For example, exposure to dying worms may be dependent on the natural parasite life-span in un-treated populations and therefore particularly limited in young children with a short history of exposure (Woolhouse and Hagan 1999; Mutapi *et al.* 2008), but treatment may accelerate exposure to these antigens making the immune responses of children more 'adult-like' (Grogan *et al.* 1996b; Mutapi *et al.* 2003).

It is interesting that I have identified an association between egg-specific cytokine responses and resistance to re-infection since egg-specific immune responses have been described as 'fast evolving' (Mutapi *et al.* 1999) and 'short-lived' (Woolhouse and Hagan 1999) since their abundant carbohydrate antigens elicit high titres of IgM (Mutapi *et al.* 1999) and cannot

be directly presented to the T cell receptor via MHC II. Pro-inflammatory glycan-specific responses to both eggs and cercariae, which are also enriched with carbohydrate molecules (Samuelson and Caulfield 1985; Jang-Lee *et al.* 2007), have also been proposed to act as 'decoy' responses that limit the development of peptide-specific effector and memory responses (Eberl *et al.* 2001; Kariuki *et al.* 2008). However, in light of my observations, it is also possible that immune cross-reactivity between cercariae and egg glycans (Jang-Lee *et al.* 2007; Kariuki *et al.* 2008) are of relevance to protective immunity. For example, *in vitro* studies suggest that glycolipids from *S. mansoni* eggs, but not adult worms, induce secretion of IL-6, TNFα and IL-10 by monocytes isolated from praziquantel-treated participants endemically exposed to *S. haematobium* (van der Kleij *et al.* 2002b). Both IgM titres (Mutapi *et al.* 2003) and eosinophils counts (Ganley-Leal *et al.* 2006) have been shown to increase after praziquantel treatment. Thus, activation of innate non-T cell cytokine responses by egg antigens after treatment may alter the environment in which schistosome peptide antigens are presented to T cells (reviewed by others (Jenkins and Allen 2010)) and immune cross-reactivity, particularly between cercariae and eggs (Jang-Lee *et al.* 2007; Kariuki *et al.* 2008), may also contribute to the development of adaptive memory responses to other life-cycle stages. Since resistance to high intensity infections takes years to develop naturally (Yazdanbakhsh and Sacks 2010) and adult worm antigens do not elicit large amounts (relative to CAP or SEA) of any of the cytokines measured before or after treatment (chapter 3 and 5), treatment may be required to trigger this process and overcome tolerance of infection. This assertion is supported by observations that PBMC proliferative responses to *S. mansoni* cercariae and egg antigens, but not adult worms, were consistently lower in individuals who were subsequently re-infected than in individuals who did not acquire new infections up to 2 years after treatment (Colley *et al.* 1986). Mathematical models also predict that immune responses elicited by one life-cycle stage, including eggs, and targeting another could induce protective immunity in such a way as to replicate the distribution of infection by age frequently observed in schistosome-endemic communities prior to treatment (Woolhouse 1994; Mitchell *et al.* 2008).

Clearly both the time after treatment at which cytokine responses were assessed and the duration of whole blood antigen re-stimulation in culture are important considerations for interpreting whether cytokine profiles are related to anti-parasite immunity after treatment. For example, previous human schistosome studies have used different culture durations for different cytokines to assess individual responses at their peak level (van den Biggelaar *et al.* 2002; Joseph *et al.* 2004a; Joseph *et al.* 2004b), whereas I have chosen to measure all

cytokines at a single timepoint to allow characterisation of the cytokine profile as a whole. The advantage of my approach is that it enables simultaneous interactions between cytokines in cultured whole blood to be characterised, which are not biased by the effect of different culture duration. Furthermore, direct comparisons with other studies are made with caution since for those investigating re-infection over a longer period (e.g. 2 years (van den Biggelaar *et al.* 2002) and 21 months (Caldas *et al.* 2000)), individuals who appeared resistant to re-infection in our study (i.e. 'un-infected' at 18 months post-treatment) may not qualify as such due to re-infection occurring at later post-treatment timepoints.

8.4 Are glutathionine-S-transferase-specific cytokine responses associated with anti-parasite immunity?

Although *S. haematobium* GST is considered to be the most promising of the vaccine candidate antigens against human schistosomiasis (Capron *et al.* 2002) I have not identified an association between the cytokine profile elicited by GST and resistance to high intensity infections before treatment (chapter 4) or a reduced risk of re-infection after treatment (chapter 6). Furthermore, I did not identify the strong gender-dependent variation in immune responses to GST noted elsewhere (Remoué *et al.* 2000; Remoué *et al.* 2001), although this may be due to inclusion of a broad age-range (5-84 years) where previous studies restricted their analyses to older children and adults (aged 12 years and above (Remoué *et al.* 2000) and aged 35 years and above (Remoué *et al.* 2001)). Thus, what may have been over-looked in these previous studies is the age-dependent cytokine response to GST, which has also been observed in antibody responses to GST isoforms present in WWH both before (Mutapi *et al.* 2008) and after (Mutapi *et al.* 2003) treatment.

GST-specific pro-inflammatory cytokine responses are highest at the age of peak-infection intensity prior to treatment (chapter 4) and those individuals in the 11-12 year age group that bore the highest infection intensity had the lowest increase in pro-inflammatory cytokine responses to GST after treatment (chapter 6). Since GST is abundantly expressed at various stages of the schistosome life-cycle (Curwen *et al.* 2004) it is unsurprising that changes in parasite exposure (i.e. with age or following treatment) would influence GST-specific responses. Accordingly I have shown that the predominant cytokine profiles elicited by GST (e.g. pro-inflammatory (GST PC1) and regulatory/Th17 (GST PC3) (chapter 4)) correspond to those elicited by WWH and SEA and the age and treatment-dependent dynamics of GST

and SEA/WWH cytokine profiles are also similar. In particular, 11-12 year olds may have the highest pre-treatment GST responses because of their high levels of infection and these responses may decline less rapidly after treatment due to the slower decline in GST levels due to their large numbers of patent worms. Thus, these dynamics suggest that GST-specific responses more accurately indicate current infection levels than development of immunity.

It is important to note that because both pre and post-treatment protection was defined according to egg counts in urine in this study the purported effect of anti-GST responses on parasite fecundity (Boulanger *et al.* 1991) could not be investigated. Furthermore, whilst exposure to GST at the concentrations present in a typical schistosome infection may be insufficient to promote protective cytokine responses, my studies do not preclude the induction of protective GST-specific antibody responses (Grzych *et al.* 1993) or beneficial effects of GST vaccination at higher doses, which appear to induce antibody-mediated protection in animal models (Lane *et al.* 1998; Capron *et al.* 2001). In support of the latter hypothesis, the post-treatment increase in GST-specific cytokines, most notably IL-6 and IL-8, but also TNFα, IL-12p70, IL-13 and IL-23 (chapter 5), were consistent with a treatment-induced increase in the priming of effector cells by GST after treatment. Thus my results confirm the importance of treatment for boosting cytokine responses to GST only shown previously for antibody responses in human (Mutapi *et al.* 2003; Mutapi *et al.* 2005) and murine studies (Doenhoff *et al.* 1987; Dupre *et al.* 1999). Importantly, co-administration of a GST-based vaccine with praziquantel is the subject of an on-going Phase III clinical trial (NIH 2009), the first of its kind in human schistosomiasis. It is hoped that this approach will allow a more comprehensive analysis of cytokine, cellular and antibody responses to this antigen since after many years of focus on both *S. mansoni* and *S. haematobium* GST there remains little published data, but much published discussion (Capron *et al.* 1987; Capron *et al.* 2002; Capron *et al.* 2005; Wilson and Coulson 2006), on its efficacy in endemically-exposed humans.

8.5 Can short-term helminth infections limit immune-mediated pathologies during allergy?

Although natural helminth infections are currently aggregated in developing areas of Africa, Asia, and Latin America (Hotez *et al.* 2008) it is only in the relatively recent past (40-50 years (Stoll 1947; Mannino *et al.* 1998)) and as a result of dramatic improvements in

sanitation and healthcare that helminth infections have ceased to be ubiquitous in affluent, urbanised countries. Thus, notwithstanding their devastating impact on global health, the reduced prevalence of helminth infection and their immunomodulatory effects on the immune system may have had repercussions for diseases caused by immune hyper-reactivity to innocuous antigens (e.g. allergens and auto-antigens). However, the jury is still out on whether short-term experimental helminth infections can reduce allergic and auto-immune reactivity in the same way as chronic infection in an endemic setting (Leonardi-Bee *et al.* 2006; Flohr *et al.* 2009; Feary *et al.* 2011) and immunotherapeutic helminth infections are the subject of on-going clinical trials (Summers *et al.* 2005a; Summers *et al.* 2005b; Croese *et al.* 2006; Daveson *et al.* 2009a; Daveson *et al.* 2009b; Bager *et al.* 2010a; Feary *et al.* 2010; Daveson *et al.* 2011; Fleming *et al.* 2011). In chapter 7 I provide the first and largest analysis of cytokine responses to experimental *Trichuris suis* infection in helminth naïve humans and the first assessment of the effect of these infections on responses to pollen allergens during allergic airway disease.

It is clear from the results of chapter 7 that *T. suis* ova (TSO) therapy for allergic rhinitis represents a complex balance between the natural course of a GI nematode infection and, particularly for seasonally exacerbated allergy, the dynamics of the allergic response. However, despite marked changes in the cytokine environment elicited by infection and *T. suis* excretory/secretory (E/S) antigens, these did not correspond to a reduction in clinical symptoms of allergic rhinitis (Bager *et al.* 2010a). Notably, the grass pollen season was characterised by elevated Th2-type PBMC responses typical of allergic responses (Benson *et al.* 1997; Klimek *et al.* 1999; Wachholz *et al.* 2002; Scavuzzo *et al.* 2003) in infected and un-infected participants despite elevated *T. suis*-specific PBMC IL-10 and IFNɣ responses, which might be expected to regulate Th2 responses.

To-date the most successful trials of helminth infection as an immunotherapy for inflammatory disease have been seen for Crohn's disease and ulcerative colitis (Summers *et al.* 2005a; Summers *et al.* 2005b; Croese *et al.* 2006) , where infection and inflammation occur within the same locale. Since helminth-mediated suppression of allergen-specific responses may occur more readily at the site of infection than at distal allergic foci (e.g. the airways) care should also be taken when directly comparing the clinical efficacy observed in the cohort selected for the current study (Bager *et al.* 2010a) with that of previous trials. Allergic immune responses may be restricted to the site of allergen sensitisation (Denburg *et al.* 1990; Saito *et al.* 2001) and may therefore be unaffected by changes in circulating

immune cells or the gut mucosa. Crohn's disease is also perpetuated by Th1 and Th17-polarised immunopathology (Brand 2009) and these responses may be more readily altered by helminth-induced Th2-type responses than allergic disease where pathogenesis is Th2-mediated. Thus, as proposed by others (Summers *et al.* 2010), a longer duration of exposure to *T. suis* may be also be required prior to the grass pollen season in the current model than in those described previously. It is also important to note that *T. suis* treatment of the study cohort investigated in chapter 7 corresponds to a relatively short-term and primary exposure to infection and studies of natural infection with other helminth species have shown that cytokine responses in acute phase infection differ markedly to those that develop in the chronic setting (Montenegro *et al.* 1999a; Babu and Nutman 2003). Notably, a recent immunological characterisation of self-administered *T. trichiura* indicated that protection against irritable bowel syndrome (IBD) only manifested after several months-years of treatment and particularly during periods of high intensity infection (Broadhurst *et al.* 2010). However, establishment of intense and chronic infections with a natural human parasite, such as that seen in the latter study (Broadhurst *et al.* 2010), would be unlikely to meet the safety requirements of most clinical trials. Thus, despite its limitations, an adapted regime of TSO remains a more realistic option for helminth-based targeting of allergic disease, particularly in light of the regulatory cytokine responses (*T. suis*-specific IL-10 and a tendency for higher allergen-specific TGFβ responses) in the infected group relative to placebo-treated controls (chapter 7).

Alternatively, promising results from murine studies suggest that administration of helminth peptides, rather than live infections have potential as immunotherapies in humans. For example, nematode excretory/secretory products have been shown to directly reduce airway hyper-reactivity (eosinophilia, serum antibody titres, IL-4 and IL-5) if co-administered with allergen and these effects occurred in the context of elevated parasite-specific Th2 response (Trujillo-Vargas *et al.* 2007). *T. suis* E/S also elicited high levels of effector cytokines in both TSO- and placebo-treated participants (chapter 7), suggesting that E/S can effectively polarise human immune responses in the absence of infection. A helminth antigen-based approach would have the added advantage of negating the risk of the adverse side-effects during live parasite infection and the potential for combination with existing intravenous and intralymphatic allergen-based therapies with known clinical efficacy (Senti *et al.* 2005; Senti *et al.* 2008).

8.6 Is there a stereo-typical cytokine response to parasitic helminth infection in humans?

The results of this thesis have increased the range of cytokines assessed in human *S. haematobium* infection and suggest that immune responses both before and after treatment constitute a variety of cellular effector phenotypes. Interestingly, despite the generally-accepted view that helminth infections induce a stereotypical Th2-polarised immune responses, whole blood Th2-type effector cytokine responses (IL-4, IL-5 and IL-13) did not significantly vary according to *S. haematobium* infection intensity (chapter 4) and, with the exception of the decline in IL-5 responses, were not significantly affected by treatment (chapter 5) in Magaya community. In contrast, systemic IL-5 and *T. suis* specific PBMC IL-4, IL-5 and IL-13 responses were markedly elevated during experimental *T. suis* infection relative to placebo-treated controls (chapter 7). Inconsistencies between these studies, particularly the life-history of the 2 helminth species, assay of whole blood versus plasma and PBMC responses and predisposition of allergic individuals towards a Th2-polarised response, make it impossible to draw direct comparisons between them. However these observations do raise the question of whether helminth specific responses can be considered 'stereotypical' in humans.

Although I have shown that a variety of alternate effector cytokines are simultaneously elicited and these responses vary more markedly than Th2-type cytokines between schistosome antigens (chapter 3), according to host age and infection intensity (chapter 4), my results clearly do not preclude the importance of the Th2 phenotype during *S. haematobium* infection. For example, although whole blood Th2-type cytokines were not detected at high levels relative to previous observations in a schistosome hyper-endemic area (Joseph *et al.* 2004a; Joseph *et al.* 2004b), the changes observed after treatment in the current study (chapter 5) suggest that a Th2-polarised environment is relatively more predominant in peripheral whole blood responses to *S. haematobium* SEA and WWH during infection (chapter 3). However, a variety of studies suggest that levels of different immune markers vary according to the duration of helminth infection (Ottesen *et al.* 1978; Colebunders *et al.* 1995; Montenegro *et al.* 1999a; Barbosa *et al.* 2001; Caldas *et al.* 2008; de Morais *et al.* 2008), local transmission intensity(Mutapi *et al.* 1997, 1999; Scott *et al.* 2001; Mduluza *et al.* 2003), age (Day *et al.* 1991b; Agnew *et al.* 1996; Fulford *et al.* 1998; Faulkner *et al.* 2002; Mutapi *et al.* 2003; Mutapi *et al.* 2008; de Moira *et al.* 2010), gender (Webster *et al.*

1997b; Remoué *et al.* 2001), antigen-specificity (Joseph *et al.* 2004a; van der Kleij *et al.* 2004; Everts *et al.* 2009) and co-infection (Ganley-Leal *et al.* 2006; Geiger 2008; Wilson *et al.* 2008; Diallo *et al.* 2010) among other factors, and thus the immune response to human helminth infection is often described as a 'mixed' phenotype, consistent with my observations. Furthermore, the distinct life-histories of GI parasites, such as *T. suis*, and tissue-dwelling parasites, such as *S. haematobium*, may lead to differences in the immune responses they elicit (Bourke *et al.* 2011). The most widely cited evidence for direct Th2-polarisation of human cells by helminth antigens comes from observations in cell-specific cultures from helminth-naïve humans (Schramm *et al.* 2003; Everts *et al.* 2009), which may belie the role of alternate cell types and the effect of long-term conditioning by endemic exposure from birth. Genetic studies also suggest that polymorphisms in the 5q31-q33 region, where Th2-type cytokine genes are located, is associated with resistance to infection across a range of human populations and for both *S. mansoni* and *S. haematobium*, but resistance to peri-portal fibrosis in the liver is associated with polymorphisms in IFNγ (Dessein *et al.* 2004). Thus, protective immunity (comprising both anti-parasite and anti-pathology responses) may require development of compartmentalised responses within individuals and 'mixed' cytokine responses may be genetically selected for in helminth-exposed human populations. Evidence for the latter comes from population genetic analyses indicating that exposure to a diverse range of micro and macro-parasitic species has promoted variability in human interleukin genes across 52 human populations (Fumagalli *et al.* 2009). Murine models of helminth infection also highlight the importance of Th2-type responses in the tissues where parasites are present (Cook *et al.* 2011; Jenkins *et al.* 2011) and suggest that sensitivity to IL-4 may be relatively limited in the periphery (Perona-Wright *et al.* 2010).

Although local immune responses can be investigated in humans, for example via collection of gut biopsies (Broadhurst *et al.* 2010), collection of these samples is invasive, time-consuming and requires technical equipment that is unavailable during most field studies in endemic areas. Thus a challenge for researchers of both human infections and animal models is to identify how peripheral immune markers relate to the mechanisms of protection and the clinical features of disease. The first step for human studies is to address whether novel cellular effectors identified in laboratory infections are detectable in peripheral samples and vary during natural helminth infections, as I have done for Th17-type cytokines in urinary schistosomiasis (chapters 3-6). Testing these paradigms will enable both shared

characteristics of the immune responses to different parasite species and the differences between them to be identified in future studies.

8.8 Future prospects

Due to the breadth of the immunoepidemiological field study of which chapters 3-6 are constituent parts, there are several questions arising from this thesis that can be immediately addressed. Firstly, an important consideration is how the secreted cytokine profiles observed can be more directly linked to the phenotypes of cultured whole blood cells. This could be achieved by measuring the expression of transcription factors associated with different cellular effector phenotypes in cultured whole blood cells. Of particular interest would be expression of T-bet (Th1), GATA3 (Th2) and RORC2 (the human orthologue of RORγt , the characteristic transcription factor driving murine Th17 differentiation (Unutmaz 2009)) (Diaz and Allen 2007). Although mRNA expression analysis was beyond the scope of the current study, I collected and stored lymphocyte-enriched samples of cultured whole blood in RNA preservation buffer throughout the pre and 6 weeks post-treatment sampling visits to Magaya and analysis of these samples will allow the correspondence between cytokine profiles and molecular markers of cellular phenotype to be compared. Another means of relating cytokine responses to cellular effector responses would be to phenotype PBMCs (via surface receptor expression, schistosome-specific proliferation assays and intracellular cytokine staining) isolated from the same individuals. Analyses of purified monocytes, granulocytes and various effector B and T cell subsets present in PBMC isolates from Magaya community are being conducted by my fellow researchers at the University of Edinburgh. The latter will also allow the relationship between these cell types and age and infection intensity to be explored as I have done for whole blood cytokine profiles in chapters 4 and 6.

In order to validate the observed association between post-treatment schistosome-specific cytokine profiles and the risk of subsequent re-infection discussed in chapter 6, it will also be important to assess the duration of treatment-induced changes in these responses beyond 6 weeks. The latter can be directly assessed using supernatants I have collected from whole blood cultures conducted 6 months after praziquantel treatment of Magaya community. Although, previous studies have assessed immune responses over longer post-treatment periods (Colley *et al.* 1986; van den Biggelaar *et al.* 2002), analysis of these samples would

provide the most comprehensive longitudinal assessment of schistosome-specific cytokine responses to date and add greatly to our understanding of the longer-term implications of treatment on the development of protective immune profiles.

The results of this thesis also highlight several important areas of investigation for future studies. In particular, the limited available sample volumes from many participants meant that fewer CAP-stimulated whole blood cultures were conducted than for SEA and WWH meaning that the age range and infection ranges were insufficient to conduct an age-structured analysis of cercariae-specific cytokine responses. The results presented in chapters 3 and 5 suggest that immune responses targeting cercariae warrant further study. More specifically I would advocate regular inclusion of CAP in whole blood culture studies conducted in the field and a more detailed comparison of which constituent molecules of CAP are recognised by serum antibodies both before and after treatment (as has been conducted for WWH (Mutapi *et al.* 2005; Mutapi *et al.* 2008; Mutapi *et al.* 2011a).

The relative contribution of different parasite antigens to the increase in post-treatment cytokine responses identified in chapter 5 could be explored in more detail in future studies by assaying changes in the levels of circulating antigen in sera or urine. Of specific relevance to the increase in SEA-specific cytokine responses I observed 6 weeks after treatment (chapter 5), previous studies indicate that egg antigens continue to be detected up to 6 weeks after treatment (Nibbeling *et al.* 1998) and thus may have a prolonged impact on the post-treatment cytokine environment. Circulating antigen assays might also allow inferences to be made as to whether treatment influences parasite fecundity (Agnew *et al.* 1996; van Lieshout *et al.* 1998) and might be more sensitive to low intensity infections than urine egg counts.

Importantly, the absence of data on the clinical morbidity in Magaya community also means that my observations cannot be related to the development of anti-pathology immunity during schistosomiasis. Thus an important extension to future studies would be to quantify markers of host pathology (e.g. ultrasound evaluation of the bladder, liver and spleen, assessment of proteinurea and haematuria) and investigate the relationship between these markers and the schistosome-specific cytokine profile. There are already a number of cross-sectional studies suggesting that the balance between different effector cytokines influences immunopathology during schistosomiasis (Wamachi *et al.* 2004; Wilson *et al.* 2008) and, in

light of my observations, it will be particularly important to test whether the role of Th17-type cytokines in murine pathology (Rutitzky *et al.* 2008; Rutitzky *et al.* 2009) is also evident in human disease.

During future field studies, it will continue to be important to investigate whether immune mechanisms identified during experimental helminth infections translate into detectable patterns in natural human infections. In particular, since the effector function of secreted cytokines relies on the expression of the appropriate receptors (discussed above), assaying cytokine receptor expression in cells isolated from the human periphery would be a useful complement to quantifying cytokine production and this has not been assessed in human field studies or clinical trials of helminth infection to-date. For example, the latter may yield insights into why different Th2-type cytokines dissociate in response to schistosome antigen stimulation as observed in the current study and by others (Grogan *et al.* 1998b; Scott *et al.* 2000).

Clinical trials of experimental helminth infection in humans also provide a rare opportunity to investigate how helminth infections can modify their local immune environment and may impact upon immunopathology in auto-immune (Broadhurst *et al.* 2010) and allergic disease (Benson *et al.* 1997; Scavuzzo *et al.* 2003). Thus collection of samples for immunological analyses should be a key consideration in the design of future studies and an important means of assessing the immunological impact of helminth-based therapies. An immediate extension to chapter 7 would be to assay IL-2, IL-6, IL-8, IL-12p70, IL-17A, IL-21 and IL-23 in the plasma and PBMC supernatant samples from the clinical trial of TSO therapy. This would provide a more complete cytokine analysis of treated individuals relative to placebo controls and is of particular interest in light of recent data showing that *Trichuris trichuria* can therapeutically alter GI IL-17-production during inflammatory bowel disease (Broadhurst *et al.* 2010).

8.9 General conclusions

The major conclusion from this thesis is that cytokine profiles elicited by parasitic helminths and their antigens are influenced by variations in the human host, parasite life-history and their shared environment. This was evident in a community endemically-exposed to *S. haematobium* where cytokine responses were influenced by host sex, age and exposure

history, parasite cycle stage and infection intensity and anti-helminthic treatment. In allergic rhinitis sufferer's experimentally infected with *T. suis* cytokine responses were affected both by infection and by temporal variations in environmental pollen allergens. Characterising immune heterogeneity is essential for understanding how immune responses to helminth infection can develop in different contexts.

As we learn more about the complexity of the human immune response to infection, it is becoming increasingly important to find meaningful ways of identifying and interpreting patterns of these responses. The results of this thesis highlight both the importance of assaying a greater range of cytokine responses during human field studies and the usefulness of analysing cytokines as co-incident and interacting immune responses rather than in isolation. This integrated approach provides a powerful means of relating the immune phenotype elicited by parasite antigens in the periphery to epidemiological patterns of natural helminth infection and an effective means of assessing the immunological impact of experimental infection on immune-mediated disease.

References

Abe, M., Harpel, J. G., Metz, C. N., Nunes, I., Loskutoff, D. J. and Rifkin, D. B. (1994). An assay for transforming growth factor-beta using cells transfected with a plasminogen-activator inhibitor-1 promoter luciferase construct. Analytical Biochemistry 216(2): 276-284.

Ackerman, S. J., Gleich, G. J., Loegering, D. A., Richardson, B. A. and Butterworth, A. E. (1985). Comparative toxicity of purified human eosinophil granule cationic proteins for schistosomula of *Schistosoma mansoni*. American Journal of Tropical Medicine and Hygiene 34(4): 735-745.

Acosta, L. P., McManus, D. P., Aligui, G. D. L., Olveda, R. M. and Tiu, W. U. (2004). Antigen-specific antibody isotype patterns to *Schistosoma japonicum* recombinant and native antigens in a defined population in Leyte, The Philippines. American Journal of Tropical Medicine and Hygiene 70(5): 549-555.

Agnew, A., Fulford, A. J. C., Mwanje, M. T., Gachuhi, K., Gutsmann, V., Krijger, F. W., Sturrock, R. F., Vennervald, B. J., Ouma, J. H., Butterworth, A. E. and Deelder, A. M. (1996). Age-dependent reduction of schistosome fecundity in *Schistosoma haematobium* but not *Schistosoma mansoni* infections in humans. American Journal of Tropical Medicine Hygeine 55(3): 338-343.

Agnew, A. M., Murare, H. M. and Doenhoff, M. J. (1989a). Specific cross-protection between *Schistosoma bovis* and *S. haematobium* induced by highly irradiated infections in mice. Parasite Immunology 11(4): 341-349.

Agnew, A. M., Murare, H. M., Lucas, S. B. and Doenhoff, M. J. (1989b). *Schistosoma bovis* as an immunological analogue of *S. haematobium*. Parasite Immunology 11(4): 329-340.

Agnew, A. M., Murare, H. M. and Doenhoff, M. J. (1993). Immune attrition of adult schistosomes. Parasite Immunology 15(5): 261-271.

Ahmed, R. and Gray, D. (1996). Immunological memory and protective immunity: Understanding their relation. Science 272(5258): 54-60.

Ahmed, S. F., Oswald, I. P., Caspar, P., Hieny, S., Keefer, L., Sher, A. and James, S. L. (1997). Developmental differences determine larval susceptibility to nitric oxide-mediated killing in a murine model of vaccination against *Schistosoma mansoni*. Infection and Immunity 65(1): 219-226.

Akira, S., Uematsu, S. and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell 124(4): 783-801.

Al-Sherbiny, M., Osman, A., Barakat, R., El Morshedy, H., Bergquist, R. and Olds, R. (2003). *In vitro* cellular and humoral responses to *Schistosoma mansoni* vaccine candidate antigens. Acta Tropica 88(2): 117-130.

Allen, J. E. and Maizels, R. M. (1997). Th1-Th2: reliable paradigm or dangerous dogma? Immunology Today 18(8): 387-392.

Allen, J. E. and Maizels, R. M. (2011). Diversity and dialogue in immunity to helminths. Nature Reviews Immunology 11(6): 375-388.

Anderson, R. M. and May, R. M. (1992). Part II: Macroparasites. Infectious diseases of humans: dynamics and control, Oxford University Press**:** 433 - 656.

Andrews, P. (1985). Praziquantel: mechanisms of anti-schistosomal activity. Pharmacology and Therapeutics 29(1): 129-156.

Angeli, V., Faveeuw, C., Roye, O., Fontaine, J., Teissier, E., Capron, A., Wolowczuk, I., Capron, M. and Trottein, F. (2001). Role of the parasite-derived prostaglandin D2 in the inhibition of epidermal Langerhans cell migration during schistosomiasis infection. Journal of Experimental Medicine 193(10): 1135-1147.

Appleton, C. C. (1984). Schistosome dermatitis - an unrecognized problem in South Africa. South African Medical Journal 65(12): 467-469.

Asher, M. I., Montefort, S., Björkstén, B., Lai, C. K. W., Strachan, D. P., Weiland, S. K. and Williams, H. (2006). Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases I and III repeat multicountry cross-sectional surveys. Lancet 368(9537): 733-743.

Ashton, P. D., Harrop, R., Shah, B. and Wilson, R. A. (2001). The schistosome egg: development and secretions. Parasitology 122: 329-338.

Babu, S. and Nutman, T. B. (2003). Proinflammatory cytokines dominate the early immune response to filarial parasites. Journal of Immunology 171(12): 6723-6732.

Babu, S., Bhat, S. Q., Kumar, N. P., Lipira, A. P., Kumar, S., Karthick, C., Kumaraswami, V. and Nutman, T. B. (2009). Filarial lymphedema is characterized by antigen-specific Th1 and Th17 proinflammatory responses and a lack of regulatory T cells. PLoS Neglected Tropical Diseases 3(4): 1-9.

Bager, P., Arnved, J., Ronborg, S., Wohlfahrt, J., Poulsen, L. K., Westergaard, T., Petersen, H. W., Kristensen, B., Thamsborg, S., Roepstorff, A., Kapel, C. and Melbye, M. (2010a). *Trichuris suis* ova therapy for allergic rhinitis: A randomized, double-blind, placebo-controlled clinical trial. Journal of Allergy and Clinical Immunology 125(1): 123-130.

Bager, P., Wohlfahrt, J., Kristensen, B., Paulsen, L. K. and Melbye, M. (2010b). Looking into the future of *Trichuris suis* therapy Reply. Journal of Allergy and Clinical Immunology 125(3): 768-769.

Bager, P., Kapel, C., Roepstorff, A., Thamsborg, S. M., Arnved, J., Rønborg, S., Kristensen, B., Poulsen, L. K., Wohlfahrt, J. and Melbye, M. (in press). Symptoms after ingestion of pig whipworm *Trichuris suis* eggs in a randomized placebo-controlled double-blind clinical trial. PLoS ONE.

Balloul, J. M., Pierce, R. J., Grzych, J. M. and Capron, A. (1985). *In vitro* synthesis of a 28 kilodalton antigen present on the surface of the schistosomulum of *Schistosoma mansoni*. Molecular and Biochemical Parasitology 17(1): 105-114.

Barbosa, C. S., Montenegro, S. M. L., Abath, F. G. C. and Domingues, A. L. C. (2001). Specific situations related to acute schistosomiasis in Pernambuco, Brazil. Memorias Do Instituto Oswaldo Cruz 96: 169-172.

Barsoum, I. S., Gamil, F. M., Alkhafif, M. A., Ramzy, R. M., Elalamy, M. A. and Colley, D. G. (1982). Immune responses and immunoregulation in relation to human schistosomiasis in Egypt .1. Effect of treatment on *in vitro* cellular responsiveness. American Journal of Tropical Medicine and Hygiene 31(6): 1181-1187.

Barton, B. E. (1996). The biological effects of interleukin 6. Medicinal Research Reviews 16(1): 87-109.

BDBiosciences (2009). BD Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit instruction manual, Benton Dickinson and Company.

Becker, B., Mehlhorn, H., Andrews, P., Thomas, H. and Eckert, J. (1980). Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (trematoda) *in vitro*. Parasitology Research 63(2): 113-128.

Beisler, G. K., Matsuda, H., Nakao, M. and Tanaka, H. (1984). Identity of antigens in adult tegument, gut, egg and cercaria of *Schistosoma japonicum* by immunofluorescent study. Japanese Journal of Experimental Medicine 54(3): 125-130.

Benson, M., Strannegård, I. L., Wennergren, G. and Strannegård, Ö. (1997). Cytokines in nasal fluids from school children with seasonal allergic rhinitis. Pediatric Allergy and Immunology 8(3): 143-149.

Bensted-Smith, R., Anderson, R. M., Butterworth, A. E., Dalton, P. R., Kariuki, H. C., Koech, D., Mugambi, M., Ouma, J. H., Siongok, T. K. A. and Sturrock, R. F. (1987). Evidence for predisposition of individual patients to reinfection with *Schistosoma mansoni* after treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 81(4): 651-654.

Berberian, D. A., Paquin, H. O., Jr. and Fantauzzi, A. (1953). Longevity of *Schistosoma hematobium* and *Schistosoma mansoni*: observations based on a case. Journal of Parasitology 39(5): 517-519.

Bergquist, N. R. and Colley, D. G. (1998). Schistosomiasis vaccine:research to development. Parasitology Today 14(3): 99-104.

Bergquist, R., Al-Sherbiny, M., Barakat, R. and Olds, R. (2002). Blueprint for schistosomiasis vaccine development. Acta Tropica 82(2): 183-192.

Berriman, M., Haas, B. J., LoVerde, P. T., Wilson, R. A., Dillon, G. P., Cerqueira, G. C., Mashiyama, S. T., Al-Lazikani, B., Andrade, L. F., Ashton, P. D., Aslett, M. A., Bartholomeu, D. C., Blandin, G., Caffrey, C. R., Coghlan, A., Coulson, R., Day, T. A., Delcher, A., DeMarco, R., Djikeng, A., Eyre, T., Gamble, J. A., Ghedin, E., Gu, Y., Hertz-Fowler, C., Hirai, H., Hirai, Y., Houston, R., Ivens, A., Johnston, D. A., Lacerda, D., Macedo, C. D., McVeigh, P., Ning, Z. M., Oliveira, G., Overington, J. P., Parkhill, J., Pertea, M., Pierce, R. J., Protasio, A. V., Quail, M. A., Rajandream, M. A., Rogers, J., Sajid, M., Salzberg, S. L., Stanke, M., Tivey, A. R., White, O., Williams, D. L., Wortman, J., Wu, W. J., Zamanian, M., Zerlotini, A., Fraser-Liggett, C. M., Barrell, B. G. and El-Sayed, N. M. (2009). The genome of the blood fluke *Schistosoma mansoni*. Nature 460(7253): 352-U365.

Bethony, J., Williams, J. T., Blangero, J., Kloos, H., Gazzinelli, A., Soares, B., Coelho, L., Alves-Fraga, L., Williams-Blangero, S., Loverde, P. T. and Correa-Oliveira, R. (2002). Additive host genetic factors influence fecal egg excretion rates during *Schistosoma mansoni* infection in a rural area in Brazil. American Journal of Tropical Medicine and Hygiene 67(4): 336-343.

Black, C. L., Steinauer, M. L., Mwinzi, P. N. M., Secor, W. E., Karanja, D. M. S. and Colley, D. G. (2009). Impact of intense, longitudinal retreatment with praziquantel on cure rates of schistosomiasis mansoni in a cohort of occupationally exposed adults in western Kenya. Tropical Medicine and International Health 14(4): 450-457.

Black, C. L., Mwinzi, P. N., Muok, E. M., Abudho, B., Fitzsimmons, C. M., Dunne, D. W., Karanja, D. M., Secor, W. E. and Colley, D. G. (2010). Influence of exposure history on the immunology and development of resistance to human Schistosomiasis mansoni. PLoS Neglected Tropical Diseases 4(3): e637.

Blount, D., Hooi, D., Feary, J., Venn, A., Telford, G., Brown, A., Britton, J. and Pritchard, D. (2009). Immunologic profiles of persons recruited for a randomized, placebo-controlled clinical trial of hookworm infection. American Journal of Tropical Medicine and Hygiene 81(5): 911-916.

Booth, M., Mwatha, J. K., Joseph, S., Jones, F. M., Kadzo, H., Ireri, E., Kazibwe, F., Kemijumbi, J., Kariuki, C., Kimani, G., Ouma, J. H., Kabatereine, N. B., Vennervald, B. J. and Dunne, D. W. (2004). Periportal fibrosis in human *Schistosoma mansoni* infection is associated with low IL-10, low IFN-gamma, high TNF-alpha, or low RANTES, depending on age and gender. Journal of Immunology 172(2): 1295-1303.

Borish, L., Aarons, A., Rumbyrt, J., Cvietusa, P., Negri, J. and Wenzel, S. (1996). Interleukin-10 regulation in normal subjects and patients with asthma. Journal of Allergy and Clinical Immunology 97(6): 1288-1296.

Boulanger, D., Reid, G. D., Sturrock, R. F., Wolowczuk, I., Balloul, J. M., Grezel, D., Pierce, R. J., Otieno, M. F., Guerret, S., Grimaud, J. A., Butterworth, A. and Capron, A. (1991). Immunization of mice and baboons with the recombinant Sm28GST affects both worm viability and fecundity after experimental infection with *Schistosoma mansoni*. Parasite Immunology 13(5): 473-490.

Boulanger, D., Warter, A., Trottein, F., Mauny, F., Bremond, P., Audibert, F., Couret, D., Kadri, S., Godin, C., Sellin, E., Pierce, R. J., Lecocq, J. P., Sellin, B. and Capron, A. (1995). Vaccination of Patas monkeys experimentally infected with *Schistosoma haematobium* using a recombinant Glutathione-S-Transferase cloned from *Schistosoma mansoni*. Parasite Immunology 17(7): 361-369.

Boulanger, D., Warter, A., Sellin, B., Lindner, V., Pierce, R. J., Chippaux, J. P. and Capron, A. (1999). Vaccine potential of a recombinant glutathione S-transferase cloned from *Schistosoma haematobium* in primates experimentally infected with an homologous challenge. Vaccine 17(4): 319-326.

Bourke, C. D., Maizels, R. M. and Mutapi, F. (2011). Acquired immune heterogeneity and its sources in human helminth infection. Parasitology 138(2): 139-159.

Brand, S. (2009). Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. Gut 58(8): 1152-1167.

Brindley, P. J. and Sher, A. (1987). The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. Journal of Immunology 139(1): 215-220.

Broadhurst, M. J., Leung, J. M., Kashyap, V., McCune, J. M., Mahadevan, U., McKerrow, J. H. and Loke, P. (2010). IL-22+ CD4+ T Cells are associated with therapeutic *Trichuris trichiura* infection in an ulcerative colitis patient. Science Translational Medicine 2(60): 60-88.

Brooker, S., Clements, A. C., Hotez, P. J., Hay, S. I., Tatem, A. J., Bundy, D. A. and Snow, R. W. (2006). The co-distribution of *Plasmodium falciparum* and hookworm among African schoolchildren. Malaria Journal 5: 99.

Brunet, L. R., Finkelman, F. D., Cheever, A. W., Kopf, M. A. and Pearce, E. J. (1997). IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. Journal of Immunology 159(2): 777-785.

Butterworth, A. E., Sturrock, R. F., Houba, V. and Rees, P. H. (1974). Antibody-dependent cell-mediated damage to schistosomula *in vitro*. Nature 252(5483): 503-505.

Butterworth, A. E., Sturrock, R. F., Houba, V., Mahmoud, A. A. F., Sher, A. and Rees, P. H. (1975). Eosinophils as mediators of antibody-dependent damage to schistosomula. Nature 256(5520): 727-729.

Butterworth, A. E., Capron, M., Cordingley, J. S., Dalton, P. R., Dunne, D. W., Kariuki, H. C., Kimani, G., Koech, D., Mugambi, M., Ouma, J. H., Prentice, M. A., Richardson, B. A., Siongok, T. K. A., Sturrock, R. F. and Taylor, D. W. (1985). Immunity after treatment of human schistosomiasis mansoni .2. Identification of resistant Individuals and analysis of their immune responses. Transactions of the Royal Society of Tropical Medicine and Hygiene 79(3): 393-408.

Caldas, I. R., Correa-Oliveira, R., Colosimo, E., Carvalho, O. S., Massara, C. L., Colley, D. G. and Gazzinelli, G. (2000). Susceptibility and resistance to *Schistosoma mansoni* reinfection: Parallel cellular and isotypic immunologic assessment. American Journal of Tropical Medicine and Hygiene 62(1): 57-64.

Caldas, I. R., Campi-Azevedo, A. C., Oliveira, L. F. A., Silveira, A. M. S., Oliveira, R. C. and Gazzinelli, G. (2008). Human schistosomiasis mansoni: Immune responses during acute and chronic phases of the infection. Acta Tropica 108(2-3): 109-117.

Capron, A., Dessaint, J. P., Capron, M., Ouma, J. H. and Butterworth, A. E. (1987). Immunity to schistosomes: progress toward vaccine. Science 238(4830): 1065-1072.

Capron, A., Capron, M., Dombrowicz, D. and Riveau, G. (2001). Vaccine strategies against schistosomiasis: From concepts to clinical trials. International Archives of Allergy and Immunology 124(1-3): 9-15.

Capron, A., Capron, M. and Riveau, G. (2002). Vaccine development against schistosomiasis from concepts to clinical trials. British Medical Bulletin 62: 139-148.

Capron, A., Riveau, G., Capron, M. and Trottein, F. (2005). Schistosomes: the road from host-parasite interactions to vaccines in clinical trials. Trends in Parasitology 21(3): 143-149.

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. and Williamson, B. (1975). Endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences of the United States of America 72(9): 3666-3670.

Celada, A. and Schreiber, R. D. (1985). Demonstration of a specific interferon-gamma (IFN-gamma) receptor on human monocytes (Mo), U937, and Hl60. Federation Proceedings 44(5): 1698-1698.

Chandiwana, S. K. (1989). The problem and control of gastrointestinal helminthiases in Zimbabwe. European Journal of Epidemiology 5(4): 507-515.

Chandiwana, S. K. and Woolhouse, M. E. J. (1991). Heterogeneities in water contact patterns and the epidemiology of *Schistosoma haematobium*. Parasitology 103: 363-370.

Chandiwana, S. K., Woolhouse, M. E. J. and Bradley, M. (1991). Factors affecting the intensity of reinfection with *Schistosoma haematobium* following treatment with praziquantel. Parasitology 102: 73-83.

Cheever, A. W., Kamel, I. A., Elwi, A. M., Mosimann, J. E. and Danner, R. (1977). *Schistosoma mansoni* and *S. haematobium* infections in Egypt: II. Quantitative parasitological findings at necropsy. American Journal of Tropical Medicine and Hygiene 26(4): 702-716.

Cheever, A. W., Duvall, R. H. and Hallack, T. A. (1983). Hepatic fibrosis in *Schistosoma haematobium* infected mice. Transactions of the Royal Society of Tropical Medicine and Hygiene 77(5): 673-679.

Chen, C. C. and Manning, A. M. (1996). TGF-beta 1, IL-10 and IL-4 differentially modulate the cytokine-induced expression of IL-6 and IL-8 in human endothelial cells. Cytokine 8(1): 58-65.

Chiaramonte, M. G., Schopf, L. R., Neben, T. Y., Cheever, A. W., Donaldson, D. D. and Wynn, T. A. (1999). IL-13 Is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by *Schistosoma mansoni* eggs. Journal of Immunology 162(2): 920-930.

Chitsulo, L., Engels, D., Montresor, A. and Savioli, L. (2000). The global status of schistosomiasis and its control. Acta Tropica 77(1): 41-51.

Christopherson, J. B. (1924). Longevity of parasitic worms: The term of living existence of *Schistosoma haematobium* in the human body. Lancet 1: 742-743.

Cluitmans, F. H., Esendam, B. H., Landegent, J. E., Willemze, R. and Falkenburg, J. H. (1994). IL-4 down-regulates IL-2-, IL-3-, and GM-CSF-induced cytokine gene expression in peripheral blood monocytes. Annals of Haematology 68(6): 293-298.

Coelho dos Santos, J. S., Menezes, C. A., Villani, F. N., Magalhaes, L. M., Scharfstein, J., Gollob, K. J. and Dutra, W. O. (2010). Captopril increases the intensity of monocyte infection by *Trypanosoma cruzi* and induces human T helper type 17 cells. Clinical and Experimental Immunology 162(3): 528-536.

Colebunders, R., Verstraeten, T., Van Gompel, A., Van den Ende, J., De Roo, A., Polderman, A. and Visser, L. (1995). Acute schistosomiasis in travelers returning from Mali. Journal of Travel Medicine 2(4): 235-238.

Colley, D. G., Barsoum, I. S., Dahawi, H. S. S., Gamil, F., Habib, M. and Elalamy, M. A. (1986). Immune responses and immunoregulation in relation to human schistosomiasis in Egypt .3. Immunity and longitudinal-studies of *in vitro* responsiveness after treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 80(6): 952-957.

Comin, F., Speziali, E., Correa-Oliveira, R. and Faria, A. M. C. (2008). Aging and immune response in chronic human schistosomiasis. Acta Tropica 108(2-3): 124-130.

Comoy, E. E., Pestel, J., Duez, C., Stewart, G. A., Vendeville, C., Fournier, C., Finkelman, F., Capron, A. and Thyphronitis, G. (1998). The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN-gamma. Journal of Immunology 160(5): 2456-2462.

Contigli, C., Silva-Teixeira, D. N., Del Prete, G., D'Elios, M. M., De Carli, M., Manghetti, M., Amedei, A., Almerigogna, F., Lambertucci, J. R. and Goes, A. M. (1999). Phenotype and cytokine profile of *Schistosoma mansoni*-specific T Cell lines and clones derived from schistosomiasis patients with distinct clinical forms. Clinical Immunology 91(3): 338-344.

Cook, P. C., Aynsley, S. A., Turner, J. D., Jenkins, G. R., Van Rooijen, N., Leeto, M., Brombacher, F. and Mountford, A. P. (2011). Multiple helminth infection of the skin causes lymphocyte hypo-responsiveness mediated by Th2 conditioning of dermal myeloid cells. PLoS Pathogens 7(3): e1001323.

Cooper, P. J., Chico, M. E., Sandoval, C., Espinel, I., Guevara, A., Kennedy, M. W., Urban, J. F., Griffin, G. E. and Nutman, T. B. (2000). Human infection with *Ascaris lumbricoides* is associated with a polarized cytokine response. Journal of Infectious Diseases 182(4): 1207-1213.

Correa-Oliveira, R., Dusse, L. M. S., Viana, I. R. C., Colley, D. G., Carvalho, O. S. and Gazzinelli, G. (1988). Human antibody responses against schistosomal antigens: I. Antibodies from patients with *Ancylostoma, Ascaris lumbricoides or Schistosoma mansoni* infections react with schistosome antigens. American Journal of Tropical Medicine and Hygiene 38(2): 348-355.

Correa-Oliveira, R., Pearce, E. J., Oliveira, G. C., Golgher, D. B., Katz, N., Bahia, L. G., Carvalho, O. S., Gazzinelli, G. and Sher, A. (1989). The human immune response to defined immunogens of *Schistosoma mansoni*: elevated antibody levels to paramyosin in stool-negative individuals from two endemic areas in Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 83(6): 798-804.

Corrêa-Oliveira, R., Malaquias, L. C. C., Falcão, P. L., Viana, I. R. C., Bahia-Oliveira, L. M. G., Silveira, A. M. S., Fraga, L. A. O., Prata, A., Coffman, R. L., Lambertucci, J. R., Cunha-Melo, J. R., Martins-Filho, O. A., Wilson, R. A. and Gazzinelli, G. (1998). Cytokines as determinants of resistance and pathology in human *Schistosoma mansoni* infection. Brazilian Journal of Medical and Biological Research 31: 171-177.

Corrigan, R. A. and Rowe, J. A. (2010). Strain variation in early innate cytokine induction by *Plasmodium falciparum*. Parasite Immunology 32(7): 512-527.

Cousins, D. J., Lee, T. H. and Staynov, D. Z. (2002). Cytokine coexpression during human Th1/Th2 cell differentiation: Direct evidence for coordinated expression of Th2 cytokines. Journal of Immunology 169(5): 2498-2506.

Coutinho, H. M., Acosta, L. P., Wu, H. W., McGarvey, S. T., Su, L., Langdon, G. C., Jiz, M. A., Jarilla, B., Olveda, R. M., Friedman, J. F. and Kurtis, J. D. (2007). Th2 cytokines are associated with persistent hepatic fibrosis in human *Schistosoma japonicum* infection. Journal of Infectious Diseases 195(2): 288-295.

Cox, F. E. G. (2002). History of human parasitology. Clinical Microbiology Reviews 15(4): 595-612.

Croese, J., O'Neil, J., Masson, J., Cooke, S., Melrose, W., Pritchard, D. and Speare, R. (2006). A proof of concept study establishing *Necator americanus* in Crohn's patients and reservoir donors. Gut 55(1): 136-137.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A. and Sedgwick, J. D. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421(6924): 744-748.

Curfs, J. H. A. J., Meis, J. F. G. M. and HoogkampKorstanje, J. A. A. (1997). A primer on cytokines: Sources, receptors, effects, and inducers. Clinical Microbiology Reviews 10(4): 742-780.

Curwen, R. S., Ashton, P. D., Johnston, D. A. and Wilson, R. A. (2004). The *Schistosoma mansoni* soluble proteome: a comparison across four life-cycle stages. Molecular and Biochemical Parasitology 138(1): 57-66.

Curwen, R. S., Ashton, P. D., Sundaralingam, S. and Wilson, R. A. (2006). Identification of novel proteases and immunomodulators in the secretions of schistosome cercariae that facilitate host entry. Molecular and Cellular Proteomics 5(5): 835-844.

Damas, P., Ledoux, D., Nys, M., Vrindts, Y., Degroote, D., Franchimont, P. and Lamy, M. (1992). Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. Annals of Surgery 215(4): 356-362.

Danso-Appiah, A., Utzinger, J., Liu, J. and Olliaro, P. (2008). Drugs for treating urinary schistosomiasis (Review). The Cochrane Library(3): 1-74.

Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R. A., Mitsdoerffer, M., Strom, T. B., Elyaman, W., Ho, I. C., Khoury, S., Oukka, M. and Kuchroo, V. K. (2008). IL-4 inhibits TGF-beta-induced Foxp3(+) T cells and, together with TGF-beta, generates IL-9(+) IL-10(+) Foxp3(-) effector T cells. Nature Immunology 9(12): 1347-1355.

Datta, S. and Sarvetnick, N. E. (2008). IL-21 Limits Peripheral Lymphocyte Numbers through T Cell Homeostatic Mechanisms. PLoS ONE 3(9): e3118.

Daveson, A. J., Jones, D. M., Gaze, S., McSorley, H., Clouston, A., Pascoe, A., Cooke, S., Speare, R., Macdonald, G. A., Anderson, R., McCarthy, J. S., Loukas, A. and Croese, J. (2011). Effect of hookworm infection on wheat challenge in Celiac Disease – A randomised double-blinded placebo controlled trial. PLoS ONE 6(3): e17366.

Daveson, A. J. M., Jones, D., Mcsorley, H., Gaze, S., Mccarthy, J., Clouston, A., Pascoe, A., Macdonald, G., Speare, R., Anderson, R., Loukas, A. and Croese, J. (2009a). A phase 2A randomized double blinded placebo controlled study evaluating immunity and gluten sensitivity by inoculating coeliac disease patients with the human hookworm *Necator americanus*. Journal of Gastroenterology and Hepatology 24: A221-A222.

Daveson, A. J. M., Jones, D., McSorley, H., Gaze, S., McCarthy, J., Clouston, A. D., Pascoe, A., Macdonald, G. A., Speare, R., Cooke, S. E., Anderson, R. P., Loukas, A. and Croese, J. (2009b). A randomized, double blinded, placebo controlled, study evaluating immunity and gluten-sensitivity by inoculating coeliac disease patients with the human hookworm *Necator americanus*. Gastroenterology 136(5): A471-A471.

David, J. R., Vadas, M. A., Butterworth, A. E., de Brito, P. A., Carvalho, E. M., David, R. A., Bina, J. C. and Andrade, Z. A. (1980). Enhanced helminthotoxic capacity of eosinophils from patients with eosinophilia. New England Journal of Medicine 303(20): 1147-1152.

Day, K. P., Gregory, W. F. and Maizels, R. M. (1991a). Age-specific acquisition of immunity to infective larvae in a bancroftian filariasis endemic area of Papua New Guinea. Parasite Immunology 13(3): 277-290.

Day, K. P., Grenfell, B., Spark, R., Kazura, J. W. and Alpers, M. P. (1991b). Age-specific patterns of change in the dynamics of *Wuchereria bancrofti* infection in Papua New Guinea. American Journal of Tropical Medicine and Hygiene 44(5): 518-527.

de Moira, A. P., Fulford, A. J. C., Kabatereine, N. B., Ouma, J. H., Booth, M. and Dunne, D. W. (2010). Analysis of complex patterns of human exposure and immunity to schistosomiasis mansoni: The influence of age, sex, ethnicity and IgE. PLoS Neglected Tropical Diseases 4(9): e820.

de Morais, C. N. L., de Souza, J. R., Melo, W. G., Aroucha, M. L., Miranda, P., Domingues, A. L. C., Abath, F. G. C. and Montenegro, S. M. L. (2008). Cytokine profile associated with chronic and acute human schistosomiasis mansoni. Memorias Do Instituto Oswaldo Cruz 103(6): 561-568.

de Waal Malefyt, R., Figdor, C. G., Huijbens, R., Mohan-Peterson, S., Bennett, B., Culpepper, J., Dang, W., Zurawski, G. and de Vries, J. E. (1993). Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. Comparison with IL-4 and modulation by IFN-gamma or IL-10. Journal of Immunology 151(11): 6370-6381.

Dean, D. A., Mangold, B. L., Harrison, R. A. and Ricciardone, M. D. (1996). Homologous and heterologous protective immunity to Egyptian strains of *Schistosoma mansoni* and *S. haematobium* induced by ultraviolet-irradiated cercariae. Parasite Immunology 18(8): 403-410.

Del Prete, G. F., De Carli, M., Mastromauro, C., Biagiotti, R., Macchia, D., Falagiani, P., Ricci, M. and Romagnani, S. (1991). Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand *in vitro* human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. Journal of Clinical Investigation 88(1): 346-350.

Demeure, C. E., Rihet, P., Abel, L., Ouattara, M., Bourgois, A. and Dessein, A. J. (1993). Resistance to *Schistosoma mansoni* in humans - Influence of the IgE/IgG4 balance and IgG2 in immunity to reinfection after chemotherapy. Journal of Infectious Diseases 168(4): 1000-1008.

Denburg, J. A., Dolovich, J., Ohtoshi, T., Cox, T., Gauldie, J. and Jordana, M. (1990). The microenvironmental differentiation hypothesis of airway inflammation. American Journal of Rhinology 4: 29-32.

deNoya, B. A., Colmenares, C., Losada, S., Fermin, Z., Masroua, G., Ruiz, L., Soto, L. and Noya, O. (1996). Do intestinal parasites interfere with the seroepidemiologic surveillance of *Schistosoma mansoni* infection? Epidemiology and Infection 116(3): 323-329.

Dessein, A., Kouriba, B., Eboumbou, C., Dessein, H., Argiro, L., Marquet, S., Elwali, N. E. M. A., Rodrigues, V., Li, Y. S., Doumbo, O. and Chevillard, C. (2004). Interleukin-13 in the skin and interferon-gamma in the liver are key players in immune protection in human schistosomiasis. Immunological Reviews 201: 180-190.

Dessein, A. J., Begley, M., Demeure, C., Caillol, D., Fueri, J., dos Reis, M. G., Andrade, Z. A., Prata, A. and Bina, J. C. (1988). Human resistance to *Schistosoma mansoni* is associated with IgG reactivity to a 37-kDa larval surface antigen. Journal of Immunology 140(8): 2727-2736.

Diallo, T. O., Remoue, F., Gaayeb, L., Schacht, A. M., Charrier, N., De Clerck, D., Dompnier, J. P., Pillet, S., Garraud, O., N'Diaye, A. A. and Riveau, G. (2010). Schistosomiasis coinfection in children influences acquired immune response against *Plasmodium falciparum* malaria antigens. PLoS ONE 5(9): e12764

Diaz, A. and Allen, J. E. (2007). Mapping immune response profiles: The emerging scenario from helminth immunology. European Journal of Immunology 37(12): 3319-3326.

Dittrich, A. M., Erbacher, A., Specht, S., Diesner, F., Krokowski, M., Avagyan, A., Stock, P., Ahrens, B., Hoffmann, W. H., Hoerauf, A. and Hamelmann, E. (2008). Helminth infection with *Litomosoides sigmodontis* induces regulatory T cells and inhibits allergic sensitization, airway inflammation, and hyperreactivity in a murine asthma model. Journal of Immunology 180(3): 1792-1799.

Doenhoff, M. J., Sabah, A. A. A., Fletcher, C., Webbe, G. and Bain, J. (1987). Evidence for an immune-dependent action of praziquantel on *Schistosoma mansoni* in mice. Transactions of the Royal Society of Tropical Medicine and Hygiene 81(6): 947-951.

Doenhoff, M. J. (1997). A role for granulomatous inflammation in the transmission of infectious disease: schistosomiasis and tuberculosis. Parasitology 115(07): 113-125.

Doetze, A., Satoguina, J., Burchard, G., Rau, T., Loliger, C., Fleischer, B. and Hoerauf, A. (2000). Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. International Immunology 12(5): 623-630.

Dolganov, G., Bort, S., Lovett, M., Burr, J., Schubert, L., Short, D., McGurn, M., Gibson, C. and Lewis, D. (1996). Coexpression of the interleukin-13 and interleukin-4 genes correlates with their physical linkage in the cytokine gene cluster on human chromosome 5q23-31. Blood 87(8): 3316-3326.

Dorresteijn, M. J., Visser, T., Cox, L. A., Bouw, M. P., Pillay, J., Koenderman, A. H., Strengers, P. F., Leenen, L. P., van der Hoeven, J. G., Koenderman, L. and Pickkers, P. (2010). C1-esterase inhibitor attenuates the inflammatory response during human endotoxemia. Critical Care Medicine 38(11): 2139-2145.

Druilhe, P., Hagan, P. and Rook, G. A. W. (2002). The importance of models of infection in the study of disease resistance. Trends in Microbiology 10(10): s38-s46.

Dunne, D. W., Richardson, B. A., Jones, F. M., Clark, M., Thorne, K. J. I. and Butterworth, A. E. (1993). The use of mouse-human chimeric antibodies to investigate the roles of different antibody isotypes, including IgA2, in the killing of *Schistosoma mansoni* schistosomula by eosinophils. Parasite Immunology 15(3): 181-185.

Dunne, D. W. and Pearce, E. J. (1999). Immunology of hepatosplenic schistosomiasis mansoni: a human perspective. Microbes and Infection 1(7): 553-560.

Dunne, D. W., Vennervald, B. J., Booth, M., Joseph, S., Fitzsimmons, C. M., Cahen, P., Sturrock, R. F., Ouma, J. H., Mwatha, J. K., Kimani, G., Kariuki, H. C., Kazibwe, F., Tukahebwa, E. and

Kabatereine, N. B. (2006). Applied and basic research on the epidemiology, morbidity, and immunology of schistosomiasis in fishing communities on Lake Albert, Uganda. Transactions of the Royal Society of Tropical Medicine and Hygiene 100(3): 216-223.

Dupre, Herv, M., Schacht, A. M., Capron, A. and Riveau, G. (1999). Control of schistosomiasis pathology by combination of Sm28GST DNA immunization and praziquantel treatment. Journal of Infectious Diseases 180(2): 454-463.

Dutra, W., Correa-Oliveira, R., Dunne, D., Cecchini, L., Fraga, L., Roberts, M., Soares-Silveira, A., Webster, M., Yssel, H. and Gollob, K. (2002). Polarized Th2 like cells, in the absence of Th0 cells, are responsible for lymphocyte produced IL-4 in high IgE-producer schistosomiasis patients. BMC Immunology 3(1): 8.

Eberl, M., Langermans, J. A. M., Vervenne, R. A., Nyame, A. K., Cummings, R. D., Thomas, A. W., Coulson, P. S. and Wilson, R. A. (2001). Antibodies to glycans dominate the host response to schistosome larvae and eggs: Is their role protective or subversive? Journal of Infectious Diseases 183(8): 1238-1247.

Ebrahim, A., El-Morshedy, H., Omer, E., El-Daly, S. and Barakat, R. (1997). Evaluation of the Kato-Katz thick smear and formol ether sedimentation techniques for quantitative diagnosis of *Schistosoma mansoni* infection. American Journal of Tropical Medicine and Hygiene 57(6): 706-708.

El Ridi, R., Ismail, S., Gaafar, T. and ElDemellawy, M. (1997). Differential responsiveness of humans with early-stage schistosomiasis haematobium to *Schistosoma haematobium* soluble adult-worm and egg antigens. Parasitology Research 83(5): 471-477.

El Ridi, R., Shoemaker, C. B., Farouk, F., El Sherif, N. H. and Afifi, A. (2001). Human T- and B-cell responses to *Schistosoma mansoni* recombinant glyceraldehyde 3-phosphate dehydrogenase correlate with resistance to reinfection with *S. mansoni* or *Schistosoma haematobium* after chemotherapy. Infection and Immunity 69(1): 237-244.

Erb, K. J. (2009). Can helminths or helminth-derived products be used in humans to prevent or treat allergic diseases? Trends in Immunology 30(2): 75-82.

Etard, J. F., Audibert, M. and Dabo, A. (1995). Age-acquired resistance and predisposition to reinfection with *Schistosoma haematobium* after treatment with praziquantel in Mali. American Journal of Tropical Medicine and Hygiene 52(6): 549-558.

Everts, B., Perona-Wright, G., Smits, H. H., Hokke, C. H., van der Ham, A. J., Fitzsimmons, C. M., Doenhoff, M. J., van der Bosch, J., Mohrs, K., Haas, H., Mohrs, M., Yazdanbakhsh, M. and Schramm, G. (2009). Omega-1, a glycoprotein secreted by *Schistosoma mansoni* eggs, drives Th2 responses. Journal of Experimental Medicine 206(8): 1673-1680.

Eyerich, S., Eyerich, K., Pennino, D., Carbone, T., Nasorri, F., Pallotta, S., Cianfarani, F., Odorisio, T., Traidl-Hoffmann, C., Behrendt, H., Durham, S. R., Schmidt-Weber, C. B. and Cavani, A. (2009). Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. Journal of Clinical Investigation 119(12): 3573-3585.

Fallon, P. G. and Dunne, D. W. (1999). Tolerization of mice to *Schistosoma mansoni* egg antigens causes elevated Type 1 and diminished Type 2 cytokine responses and increased mortality in acute infection. Journal of Immunology 162(7): 4122-4132.

Fallon, P. G. and Mangan, N. E. (2007). Suppression of T(H)2-type allergic reactions by helminth infection. Nature Reviews Immunology 7(3): 220-230.

Farah, I. O., Mola, P. W., Kariuki, T. M., Nyindo, M., Blanton, R. E. and King, C. L. (2000). Repeated exposure induces periportal fibrosis in *Schistosoma mansoni*-infected baboons: Role of TGF-beta and IL-4. Journal of Immunology 164(10): 5337-5343.

Faulkner, H., Turner, J., Kamgno, J., Pion, S. D., Boussinesq, M. and Bradley, J. E. (2002). Age and infection intensity-dependent cytokine and antibody production in human trichuriasis: the importance of IgE. Journal of Infectious Diseases 185(5): 665-672.

Feary, J., Venn, A., Brown, A., Hooi, D., Falcone, F. H., Mortimer, K., Pritchard, D. I. and Britton, J. (2009). Safety of hookworm infection in individuals with measurable airway responsiveness: a randomized placebo-controlled feasibility study. Clinical and Experimental Allergy 39(7): 1060-1068.

Feary, J., Britton, J. and Leonardi-Bee, J. (2011). Atopy and current intestinal parasite infection: a systematic review and meta-analysis. Allergy 66(4): 569-578.

Feary, J. R., Venn, A. J., Mortimer, K., Brown, A. P., Hooi, D., Falcone, F. H., Pritchard, D. I. and Britton, J. R. (2010). Experimental hookworm infection: a randomized placebo-controlled trial in asthma. Clinical and Experimental Allergy 40(2): 299-306.

Fedele, G., Spensieri, F., Palazzo, R., Nasso, M., Cheung, G. Y. C., Coote, J. G. and Ausiello, C. M. (2010). *Bordetella pertussis* commits human dendritic cells to promote a Th1/Th17 response through the activity of adenylate cyclase toxin and MAPK-pathways. PLoS ONE 5(1): e8734.

Feldmeier, H., Gastl, G. A., Poggensee, U., Daffalla, A. A., Nogueiraqueiroz, J. A., Capron, A. and Peter, H. H. (1988). Immune response in chronic schistosomiasis hematobium and mansoni - Reversibility of alterations after anti-parasitic treatment with praziquantel. Scandinavian Journal of Immunology 28(2): 147-155.

Fenwick, A., Savioli, L., Engels, D., Robert Bergquist, N. and Todd, M. H. (2003). Drugs for the control of parasitic diseases: current status and development in schistosomiasis. Trends in Parasitology 19(11): 509-515.

Fields, P. E., Kim, S. T. and Flavell, R. A. (2002). Cutting Edge: Changes in Histone Acetylation at the IL-4 and IFN-γ Loci Accompany Th1/Th2 Differentiation. The Journal of Immunology 169(2): 647-650.

Figueiredo, C. A., Barreto, M. L., Rodrigues, L. C., Cooper, P. J., Silva, N. B., Amorim, L. D. and Alcantara-Neves, N. M. (2010). Chronic intestinal helminth infections are associated with immune hyporesponsiveness and induction of a regulatory network. Infection and Immunity 78(7): 3160-3167.

Finkelman, F. D., Shea-Donohue, T., Morris, S. C., Gildea, L., Strait, R., Madden, K. B., Schopf, L. and Urban, J. F. (2004). Interleukin-4-and interleukin-13-mediated host protection against intestinal nematode parasites. Immunological Reviews 201: 139-155.

Fisher, A. C. (1934). A study of the schistosomiasis of the Stanleyville district of the Belgian congo. Transactions of the Royal Society of Tropical Medicine and Hygiene 28(3): 277-306.

Fitzpatrick, J. M., Peak, E., Perally, S., Chalmers, I. W., Barrett, J., Yoshino, T. P., Ivens, A. C. and Hoffmann, K. F. (2009). Anti-schistosomal intervention targets identified by life-cycle transcriptomic analyses. PLoS Neglected Tropical Diseases 3(11): e543

Fitzsimmons, C. M., Joseph, S., Jones, F. M., Reimert, C. M., Hoffmann, K. F., Kazibwe, F., Kimani, G., Mwatha, J. K., Ouma, J. H., Tukahebwa, E. M., Kariuki, H. C., Vennervald, B. J., Kabatereine, N. B. and Dunne, D. W. (2004). Chemotherapy for schistosomiasis in Ugandan fishermen: Treatment can cause a rapid increase in interleukin-5 levels in plasma but decreased levels of eosinophilia and worm-specific immunoglobulin E. Infection and Immunity 72(7): 4023-4030.

Fitzsimmons, C. M., McBeath, R., Joseph, S., Jones, F. M., Walter, K., Hoffmann, K. F., Kariuki, H. C., Mwatha, J. K., Kimani, G., Kabatereine, N. B., Vennervald, B. J., Ouma, J. H. and Dunne, D. W. (2007). Factors affecting human IgE and IgG responses to allergen-like Schistosoma mansoni antigens: Molecular structure and patterns of in vivo exposure. Int Arch Allergy Immunol 142(1): 40-50.

Fleming, J., Lee, J., Luzzio, C., Carrithers, M., Field, A. and Fabry, Z. (2009). A Phase-1 Trial of Probiotic Helminth Ova in Relapsing Remitting Multiple Sclerosis (RRMS). Neurology 72(11): A358-A358.

Fleming, J., Isaak, A., Lee, J., Luzzio, C., Carrithers, M., Cook, T., Field, A., Boland, J. and Fabry, Z. (2011). Probiotic helminth administration in relapsing-remitting multiple sclerosis: a phase 1 study. Multiple Sclerosis: 743-754.

Flisser, A., Elsaghier, A. A. F. and Mclaren, D. J. (1989). Effect of praziquantel on the migration and survival of developmental stages of *Schistosoma mansoni* in mice. International Journal for Parasitology 19(6): 665-672.

Flohr, C., Quinnell, R. J. and Britton, J. (2009). Do helminth parasites protect against atopy and allergic disease? Clinical and Experimental Allergy 39(1): 20-32.

Foster, C. A. (1996). VCAM-1/alpha 4-integrin adhesion pathway: therapeutic target for allergic inflammatory disorders. Journal of Allergy and Clinical Immunology 98(6 pt 2): S270-277.

Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I. and Young, I. G. (1996). Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. The Journal of Experimental Medicine 183(1): 195-201.

Fröhlich, A., Marsland, B. J., Sonderegger, I., Kurrer, M., Hodge, M. R., Harris, N. L. and Kopf, M. (2007). IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood 109(5): 2023-2031.

Fulford, A. J. C., Butterworth, A. E., Sturrock, R. F. and Ouma, J. H. (1992). On the use of age-intensity data to detect immunity to parasitic infections, with special reference to *Schistosoma mansoni* in Kenya. Parasitology 105: 219-227.

Fulford, A. J. C., Butterworth, A. E., Ouma, J. H. and Sturrock, R. F. (1995). A statistical approach to schistosome population dynamics and estimation of the life-span of *Schistosoma mansoni* in man. Parasitology 110(3): 307-316.

Fulford, A. J. C., Webster, M., Ouma, J. H., Kimani, G. and Dunne, D. W. (1998). Puberty and age-related changes in susceptibility to schistosome infection. Parasitology Today 14(1): 23-26.

Fumagalli, M., Pozzoli, U., Cagliani, R., Comi, G. P., Riva, S., Clerici, M., Bresolin, N. and Sironi, M. (2009). Parasites represent a major selective force for interleukin genes and shape the genetic predisposition to autoimmune conditions. Journal of Experimental Medicine 206(6): 1395-1408.

Galvani, A. P. (2005). Age-dependent epidemiological patterns and strain diversity in helminth parasites. Journal of Parasitology 91(1): 24-30.

Ganley-Leal, L. M., Mwinzi, P. N., Cetre-Sossah, C. B., Andove, J., Hightower, A. W., Karanja, D. M. S., Colley, D. G. and Secor, W. E. (2006). Correlation between eosinophils and protection against reinfection with *Schistosoma mansoni* and the effect of human immunodeficiency virus type 1 coinfection in humans. Infection and Immunity 74(4): 2169-2176.

Garba, A., Pion, S., Cournil, A., Milet, J., Schneider, D., Campagne, G., Chippaux, J.-P. and Boulanger, D. (2010). Risk factors for *Schistosoma haematobium* infection and morbidity in two villages with different transmission patterns in Niger. Acta Tropica 115(1-2): 84-89.

Gazzinelli, G., Katz, N., Rocha, R. S. and Colley, D. G. (1983). Immune responses during human schistosomiasis mansoni: VIII. Differential *in vitro* cellular responsiveness to adult worm and schistosomular tegumental preparations. American Journal of Tropical Medicine and Hygiene 32(2): 326-333.

Geiger, S. M. (2008). Immuno-epidemiology of *Schistosoma mansoni* infections in endemic populations co-infected with soil-transmitted helminths: present knowledge, challenges, and the need for further studies. Acta Tropica 108(2-3): 118-123.

Geisser, S. and Greenhouse, S. W. (1958). An extension of Box's results on the use of the *F* Distribution in multivariate analysis. Annals of Mathematical Statistics 29(3): 885-891.

Ghaffar, O., Laberge, S., Jacobson, M. R., Lowhagen, O., Rak, S., Durham, S. R. and Hamid, Q. (1997). IL-13 mRNA and immunoreactivity in allergen-induced rhinitis: Comparison with IL-4 expression and modulation by topical glucocorticoid therapy. American Journal of Respiratory Cell and Molecular Biology 17(1): 17-24.

Ghedin, E., Wang, S. L., Spiro, D., Caler, E., Zhao, Q., Crabtree, J., Allen, J. E., Delcher, A. L., Guiliano, D. B., Miranda-Saavedra, D., Angiuoli, S. V., Creasy, T., Amedeo, P., Haas, B., El-Sayed, N. M., Wortman, J. R., Feldblyum, T., Tallon, L., Schatz, M., Shumway, M., Koo, H., Salzberg, S. L., Schobel, S., Pertea, M., Pop, M., White, O., Barton, G. J., Carlow, C. K. S., Crawford, M. J., Daub, J., Dimmic, M. W., Estes, C. F., Foster, J. M., Ganatra, M., Gregory, W. F., Johnson, N. M., Jin, J. M., Komuniecki, R., Korf, I., Kumar, S., Laney, S., Li, B. W., Li, W., Lindblom, T. H., Lustigman, S., Ma, D., Maina, C. V., Martin, D. M. A., McCarter, J. P., McReynolds, L., Mitreva, M., Nutman, T. B., Parkinson, J., Peregrin-Alvarez, J. M., Poole, C., Ren, Q. H., Saunders, L., Sluder, A. E., Smith, K., Stanke, M., Unnasch, T. R., Ware, J., Wei, A. D., Weil, G., Williams, D. J., Zhang, Y. H., Fraser-Liggett, C., Slatko, B., Blaxter, M. L. and Scott, A. L. (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. Science 317(5845): 1756-1760.

Giboda, M. and Smith, J. M. (1994). *Schistosoma manson*i eggs as a target for praziquantel : efficacy of oral application in mice. Journal of Tropical Medicine and Hygiene 97(2): 98-102.

Gnanasekar, M., Salunkhe, A. M., Mallia, A. K., He, Y. X. and Kalyanasundaram, R. (2009). Praziquantel affects the regulatory myosin light chain of *Schistosoma mansoni*. Antimicrobial Agents and Chemotherapy 53(3): 1054-1060.

Gomez-Escobar, N., Gregory, W. F. and Maizels, R. M. (2000). Identification of tgh-2, a filarial nematode homolog of *Caenorhabditis elegans* daf-7 and human transforming growth factor beta, expressed in microfilarial and adult stages of *Brugia malayi*. Infection and Immunity 68(11): 6402-6410.

Gounni, A. S., Lamkhioued, B., Ochiai, K., Tanaka, Y., Delaporte, E., Capron, A., Kinet, J. P. and Capron, M. (1994). High affinity IgE receptor on eosinophils is involved in defense against parasites. Nature 367(6459): 183-186.

Grezel, D., Capron, M., Grzych, J. M., Fontaine, J., Lecocq, J. P. and Capron, A. (1993). Protective immunity induced in rat schistosomiasis by a single dose of the Sm28GST recombinant antigen: effector mechanisms involving IgE and IgA antibodies. European Journal of Immunology 23(2): 454-460.

Groer, M. W. and Beckstead, J. W. (2011). Multidimensional scaling of multiplex data : Human milk cytokines. Biological Research for Nursing 13(3): 289-296.

Grogan, J. L., Kremsner, P. G., Deelder, A. M. and Yazdanbakhsh, M. (1996a). Elevated proliferation and interleukin-4 release from CD4(+) cells after chemotherapy in human *Schistosoma haematobium* infection. European Journal of Immunology 26(6): 1365-1370.

Grogan, J. L., Kremsner, P. G., van Dam, G. J., Metzger, W., Mordmüller, B., Deelder, A. M. and Yazdanbakhsh, M. (1996b). Anti-schistosome IgG4 and IgE responses are affected differentially by chemotherapy in children versus adults. Journal of Infectious Diseases 173(5): 1242-1247.

Grogan, J. L., Kremsner, P. G., Deelder, A. M. and Yazdanbakhsh, M. (1998a). The effect of anti-IL-10 on proliferation and cytokine production in human schistosomiasis: fresh versus cryopreserved cells. Parasite Immunology 20(7): 345-349.

Grogan, J. L., Kremsner, P. G., Deelder, A. M. and Yazdanbakhsh, M. (1998b). Antigen-specific proliferation and interferon-gamma and interleukin-5 production are down-regulated during *Schistosoma haematobium* infection. Journal of Infectious Diseases 177(5): 1433-1437.

Gruaz, L., Delucinge-Vivier, C., Descombes, P., Dayer, J. M. and Burger, D. (2010). Blockade of T cell contact activation of human monocytes by high-density lipoproteins reveals a new pattern of cytokine and inflammatory genes. PLoS ONE 5(2): e9418.

Gryseels, B., Mbaye, A., De Vlas, S. J., Stelma, F. F., Guisse, F., Van Lieshout, L., Faye, D., Diop, M., Ly, A., Tchuem-Tchuente, L. A., Engels, D. and Polman, K. (2001). Are poor responses to praziquantel for the treatment of *Schistosoma mansoni* infections in Senegal due to resistance? An overview of the evidence. Tropical Medicine and International Health 6(11): 864-873.

Gryseels, B., Polman, K., Clerinx, J. and Kestens, L. (2006). Human schistosomiasis. Lancet 368(9541): 1106-1118.

Grzych, J., Grezel, D., Xu, C., Neyrinck, J., Capron, M., Ouma, J., Butterworth, A. and Capron, A. (1993). IgA antibodies to a protective antigen in human schistosomiasis mansoni. Journal of Immunology 150(2): 527-535.

Grzych, J. M., Pearce, E., Cheever, A., Caulada, Z. A., Caspar, P., Heiny, S., Lewis, F. and Sher, A. (1991). Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. Journal of Immunology 146(4): 1322-1327.

Grzych, J. M., De Bont, J., Liu, J., Neyrinck, J. L., Fontaine, J., Vercruysse, J. and Capron, A. (1998). Relationship of impairment of schistosome 28-kilodalton glutathione S-transferase (GST) activity to expression of immunity to *Schistosoma mattheei* in calves vaccinated with recombinant Schistosoma bovis 28-kilodalton GST. Infection and Immunity 66(3): 1142-1148.

Guidi, A., Andolina, C., Makame Ame, S., Albonico, M., Cioli, D. and Juma Haji, H. (2010). Praziquantel efficacy and long-term appraisal of schistosomiasis control in Pemba Island. Tropical Medicine and International Health 15(5): 614-618.

Hagan, P., Moore, P. J., Adjukiewicz, A. B., Greenwood, B. M. and Wilkins, H. A. (1985). *In vitro* antibody-dependent killing of schistosomula of *Schistosoma haematobium* by human eosinophils. Parasite Immunology 7(6): 617-624.

Hagan, P., Blumenthal, U. J., Dunn, D., Simpson, A. J. G. and Wilkins, H. A. (1991). Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. Nature 349(6306): 243-245.

Hagan, P. (1992). Reinfection, exposure and immunity in human schistosomiasis. Parasitology Today 8(1): 12-16.

Hammerich, L., Heymann, F. and Tacke, F. (2011). Role of IL-17 and Th17 cells in liver diseases. Clinical and Developmental Immunology 2011: 345803.

Hansell, E., Braschi, S., Medzihradszky, K. F., Sajid, M., Debnath, M., Ingram, J., Lim, K. C. and McKerrow, J. H. (2008). Proteomic analysis of skin invasion by blood fluke larvae. PLoS Neglected Tropical Diseases 2(7): e262.

Harcus, Y. M., Parkinson, J., Fernandez, C., Daub, J., Selkirk, M. E., Blaxter, M. L. and Maizels, R. M. (2004). Signal sequence analysis of expressed sequence tags from the nematode *Nippostrongylus brasiliensis* and the evolution of secreted proteins in parasites. Genome Biology 5(6).

Harnett, W. and Kusel, J. R. (1986). Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. Parasitology 93: 401-405.

Harris, A. R. C., Russell, R. J. and Charters, A. D. (1984). A review of schistosomiasis in immigrants in Western Australia, demonstrating the unusual longevity of *Schistosoma mansoni*. Transactions of the Royal Society of Tropical Medicine and Hygiene 78(3): 385-388.

Harris, C. (1967). On factors and factor scores. Psychometrika 32(4): 363-379.

Hassan, M. M., Medhat, A., Makhlouf, M. M., Shata, T., Nafeh, M. A., Osman, O. A., Deaf, E. A., Galal, N. and Fouad, Y. M. (1998). Detection of circulating antigens in patients with active *Schistosoma haematobium* infection. American Journal of Tropical Medicine and Hygiene 59(2): 295-301.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M. and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410(6832): 1099-1103.

Hellriegel, B. (2001). Immunoepidemiology - bridging the gap between immunology and epidemiology. Trends in Parasitology 17(2): 102-106.

Henri, S., Chevillard, C., Mergani, A., Paris, P., Gaudart, J., Camilla, C., Dessein, H., Montero, F., Elwali, N. E., Saeed, O. K., Magzoub, M. and Dessein, A. J. (2002). Cytokine regulation of periportal fibrosis in humans infected with *Schistosoma mansoni*: IFN-gamma is associated with protection against fibrosis and TNF-alpha with aggravation of disease. Journal of Immunology 169(2): 929-936.

Hepworth, M. R., Hamelmann, E., Lucius, R. and Hartmann, S. (2010). Looking into the future of *Trichuris suis* therapy. Journal of Allergy and Clinical Immunology 125(3): 767-768.

Herrstrom, P., Fristrom, A., Karlsson, A. and Hogstedt, B. (1997). *Enterobius vermicularis* and finger sucking in young Swedish children. Scandinavian Journal of Primary Health Care 15(3): 146-148.

Hesse, M., Piccirillo, C. A., Belkaid, Y., Prufer, J., Mentink-Kane, M., Leusink, M., Cheever, A. W., Shevach, E. M. and Wynn, T. A. (2004). The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T Cells. Journal of Immunology 172(5): 3157-3166.

Hoekstra, R., Visser, A., Otsen, M., Tibben, J., Lenstra, J. A. and Roos, M. H. (2000). EST sequencing of the parasitic nematode *Haemonchus contortus* suggests a shift in gene expression during transition to the parasitic stages. Molecular and Biochemical Parasitology 110(1): 53-68.

Hoeve, M. A., Savage, N. D. L., de Boer, T., Langenberg, D. M. L., de Waal Malefyt, R., Ottenhoff, T. H. M. and Verreck, F. A. W. (2006). Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. European Journal of Immunology 36(3): 661-670.

Hogg, K. G., Kumkate, S., Anderson, S. and Mountford, A. P. (2003a). Interleukin-12 p40 secretion by cutaneous CD11c(+) and F4/80(+) cells is a major feature of the innate immune response in mice that develop Th1-mediated protective immunity to Schistosoma mansoni. Infection and Immunity 71(6): 3563-3571.

Hogg, K. G., Kumkate, S. and Mountford, A. P. (2003b). IL-10 regulates early IL-12-mediated immune responses induced by the radiation-attenuated schistosome vaccine. International Immunology 15(12): 1451-1459.

Holm, S. (1979). A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6(2): 65-70.

Hotez, P. J., Brooker, S., Bethony, J. M., Bottazzi, M. E., Loukas, A. and Xiao, S. H. (2004). Hookworm infection. New England Journal of Medicine 351(8): 799-807.

Hotez, P. J., Brindley, P. J., Bethony, J. M., King, C. H., Pearce, E. J. and Jacobson, J. (2008). Helminth infections: the great neglected tropical diseases. Journal of Clinical Investigation 118(4): 1311-1321.

Hotez, P. J. (2009). Mass drug administration and integrated control for the world's high-prevalence neglected tropical diseases. Clinical Pharmacology & Therapeutics 85(6): 659-664.

Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T. and Paul, W. E. (1982). Identification of a T cell-derived B cell growth factor distinct from interleukin 2. Journal of Experimental Medicine 155(3): 914-923.

Huang, C. B., Altimova, Y., Strange, S. and Ebersole, J. L. (2010). Polybacterial challenge effects on cytokine/chemokine production by macrophages and dendritic cells. Inflammation Research 60(2): 119-125.

Humphreys, N. E., Xu, D., Hepworth, M. R., Liew, F. Y. and Grencis, R. K. (2008). IL-33, a potent inducer of adaptive immunity to intestinal nematodes. The Journal of Immunology 180(4): 2443-2449.

Hussein, A. H., Kaddah, M. A., Hamadto, H. H., el-Hayawan, I. A., Strickland, P. T., Abubaker, S. and Shiff, C. J. (1997). *Schistosoma mansoni*: the immune response against cercarial glycocalyx. Journal of Parasitology 83(3): 424-429.

Imai, N., Rujeni, N., Nausch, N., Bourke, C. D., Appleby, L. J., Cowan, G., Gwisai, R., Midzi, N., Cavanagh, D., Mduluza, T., Taylor, D. and Mutapi, F. (2011). Exposure, infection, systemic cytokine levels and antibody responses in young children concurrently exposed to schistosomiasis and malaria. Parasitology 138(12): 1519-1533

Jackson, J. A., Turner, J. D., Rentoul, L., Faulkner, H., Behnke, J. M., Hoyle, A., Grencis, R. K., Else, K. J., Kamgno, J., Bradley, J. E. and Boussinesq, M. (2004a). Cytokine response profiles predict species-specific infection patterns in human GI nematodes. International Journal for Parasitology 34(11): 1237-1244.

Jackson, J. A., Turner, J. D., Rentoul, L., Faulkner, H., Behnke, J. M., Hoyle, M., Grencis, R. K., Else, K. J., Kamgno, J., Boussinesq, M. and Bradley, J. E. (2004b). T helper cell type 2 responsiveness predicts future susceptibility to gastrointestinal nematodes in humans. Journal of Infectious Diseases 190(10): 1804-1811.

Jackson, J. A., Friberg, I. M., Little, S. and Bradley, J. E. (2009). Review series on helminths, immune modulation and the hygiene hypothesis: immunity against helminths and immunological phenomena in modern human populations: coevolutionary legacies? Immunology 126(1): 18-27.

Jang-Lee, J., Curwen, R. S., Ashton, P. D., Tissot, B., Mathieson, W., Panico, M., Dell, A., Wilson, R. A. and Haslam, S. M. (2007). Glycomics analysis of *Schistosoma mansoni* egg and cercarial secretions. Molecular and Cellular Proteomics 6(9): 1485-1499.

Jassim, A., Hassan, K. and Catty, D. (1987). Antibody isotypes in human schistosomiasis mansoni. Parasite Immunology 9(6): 627-650.

Jenkins, S. J., Hewitson, J. P., Ferret-Bernard, S. and Mountford, A. P. (2005a). Schistosome larvae stimulate macrophage cytokine production through TLR4-dependent and -independent pathways. International Immunology 17(11): 1409-1418.

Jenkins, S. J., Hewitson, J. P., Jenkins, G. R. and Mountford, A. P. (2005b). Modulation of the host's immune response by schistosome larvae. Parasite Immunology 27(10-11): 385-393.

Jenkins, S. J. and Mountford, A. P. (2005). Dendritic cells activated with products released by schistosome larvae drive Th2-type immune responses, which can be inhibited by manipulation of CD40 costimulation. Infection and Immunity 73(1): 395-402.

Jenkins, S. J. and Allen, J. E. (2010). Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes. Journal of Biomedicine and Biotechnology 2010: 262609.

Jenkins, S. J., Ruckerl, D., Cook, P. C., Jones, L. H., Finkelman, F. D., van Rooijen, N., MacDonald, A. S. and Allen, J. E. (2011). Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. Science 332(6035): 1284-1288.

Jepsen, K. F., Nielsen, L., Olsen, O. T., Bendtzen, K. and Poulsen, L. K. (1998). Seasonal variations in T-lymphocyte response to grass pollen allergens from pollen-allergic patients and healthy controls. Exp Clin Immunogenet 15(3): 144-153.

Jolly, E. R., Chin, C. S., Miller, S., Bahgat, M. M., Lim, K. C., DeRisi, J. and McKerrow, J. H. (2007). Gene expression patterns during adaptation of a helminth parasite to different environmental niches. Genome Biology 8(4): R65.

Jones, K. P., Morris, R. H. K., Rolf, S. and Davies, B. H. (2000). Seasonal variation in interleukin 4 patients with hayfever Cytokine 12(5): 543-545.

Joseph, S., Jones, F. M., Kimani, G., Mwatha, J. K., Kamau, T., Kazibwe, F., Kemijumbi, J., Kabatereine, N. B., Booth, M., Kariuki, H. C., Ouma, J. H., Vennervald, B. J. and Dunne, D. W. (2004a). Cytokine production in whole blood cultures from a fishing community in an area of high endemicity for *Schistosoma mansoni* in Uganda: the differential effect of parasite worm and egg antigens. Infection and Immunity 72(2): 728-734.

Joseph, S., Jones, F. M., Walter, K., Fulford, A. J., Kimani, G., Mwatha, J. K., Kamau, T., Kariuki, H. C., Kazibwe, F., Tukahebwa, E., Kabatereine, N. B., Ouma, J. H., Vennervald, B. J. and Dunne, D. W. (2004b). Increases in human T helper 2 cytokine responses to *Schistosoma mansoni* worm and worm-tegument antigens are induced by treatment with praziquantel. Journal of Infectious Diseases 190(4): 835-842.

Juszczak, M. and Glabinski, A. (2009). Th17 cells in the pathogenesis of multiple sclerosis. Advances in Hygiene and Experimental Medicine 63: 492-501.

Kahama, A. I., Kremsner, P. G., van Dam, G. J. and Deelder, A. M. (1998). The dynamics of a soluble egg antigen of *Schistosoma haematobium* in relation to egg counts, circulating anodic and cathodic antigens and pathology markers before and after chemotherapy. Transactions of the Royal Society of Tropical Medicine and Hygiene 92(6): 629-633.

Kaiser, H. F. (1960). The application of electronic computers to factor analysis. Educational and Psychological Measurement 20: 141-151.

Karanja, D. M. S., Colley, D. G., Nahlen, B. L., Ouma, J. H. and Secor, W. E. (1997). Studies on schistosomiasis in western Kenya .I. Evidence for immune-facilitated excretion of schistosome eggs from patients with S*chistosoma mansoni* and human immunodeficiency virus coinfections. American Journal of Tropical Medicine and Hygiene 56(5): 515-521.

Kariuki, T. M., Farah, I. O., Wilson, R. A. and Coulson, P. S. (2008). Antibodies elicited by the secretions from schistosome cercariae and eggs are predominantly against glycan epitopes. Parasite Immunology 30(10): 554-562.

Katz, N., Chavez, A. and Pellegring, J. (1972). A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. Revista do Instituto de Medicina Tropical de Sao Paulo 14: 397-402.

Khalife, J., Dunne, D. W., Richardson, B. A., Mazza, G., Thorne, K. J. I., Capron, A. and Butterworth, A. E. (1989). Functional role of human IgG subclasses in eosinophil-mediated killing of schistosomula of *Schistosoma mansoni*. Journal of Immunology 142(12): 4422-4427.

Khalil, N. (1999). TGF-beta: from latent to active. Microbes and Infection 1(15): 1255-1263.

King, C. H., Blanton, R. E., Muchiri, E. M., Ouma, J. H., Kariuki, H. C., Mungai, P., Magak, P., Kadzo, H., Ireri, E. and Koech, D. K. (2004). Low heritable component of risk for infection intensity and infection-associated disease in urinary schistosomiasis among Wadigo village populations in Coast Province, Kenya. American Journal of Tropical Medicine and Hygiene 70(1): 57-62.

King, C. H., Dickman, K. and Tisch, D. J. (2005). Reassessment of the cost of chronic helmintic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. Lancet 365(9470): 1561-1569.

King, C. H. (2007). Lifting the burden of schistosomiasis - Defining elements of infection-associated disease and the benefits of antiparasite treatment. Journal of Infectious Diseases 196(5): 653-655.

Kjetland, E. F., Kurewa, E. N., Mduluza, T., Midzi, N., Gomo, E., Friis, H., Gundersen, S. G. and Ndhlovu, P. D. (2010). The first community-based report on the effect of genital *Schistosoma haematobium* infection on female fertility. Fertility and Sterility 94(4): 1551-1553.

Klimek, Dormann, Jarman, Cromwell, Riechelmann and Reske, K. (1999). Short-term preseasonal birch pollen allergoid immunotherapy influences symptoms, specific nasal provocation and cytokine levels in nasal secretions, but not peripheral T-cell responses, in patients with allergic rhinitis. Clinical and Experimental Allergy 29(10): 1326-1335.

Klion, A. D. and Nutman, T. B. (2004). The role of eosinophils in host defense against helminth parasites. Journal of Allergy and Clinical Immunology 113(1): 30-37.

Kongs, A., Marks, G., Verle, P. and Van der Stuyft, P. (2001). The unreliability of the Kato-Katz technique limits its usefulness for evaluating *S. mansoni* infections. Tropical Medicine and International Health 6(3): 163-169.

Koukounari, A., Gabrielli, A. F., Toure, S., Bosque-Oliva, E., Zhang, Y. B., Sellin, B., Donnelly, C. A., Fenwick, A. and Webster, J. P. (2007). *Schistosoma haematobium* infection and morbidity before and after large-scale administration of praziquantel in Burkina Faso. Journal of Infectious Diseases 196(5): 659-669.

Kringel, H., Iburg, T., Dawson, H., Aasted, B. and Roepstorff, A. (2006). A time course study of immunological responses in *Trichuris suis* infected pigs demonstrates induction of a local type 2 response associated with worm burden. International Journal for Parasitology 36(8): 915-924.

Kringel, H. and Roepstorff, A. (2006). *Trichuris suis* population dynamics following a primary experimental infection. Veterinary Parasitology 139(1-3): 132-139.

Kruskal, J. (1964). Nonmetric multidimensional scaling: A numerical method. Psychometrika 29(2): 115-129.

Kurowska-Stolarska, M., Kewin, P., Murphy, G., Russo, R. C., Stolarski, B., Garcia, C. C., Komai-Koma, M., Pitman, N., Li, Y. B., McKenzie, A. N. J., Teixeira, M. M., Liew, F. Y. and Xu, D. M.

(2008). IL-33 induces antigen-specific IL-5(+) T cells and promotes allergic-induced airway inflammation independent of IL-4. Journal of Immunology 181(7): 4780-4790.

Lambertucci, J. R. (2010). Acute schistosomiasis mansoni: revisited and reconsidered. Memorias do Instituto Oswaldo Cruz 105(4): 422-435.

Lane, A., Boulanger, D., Riveau, G., Capron, A. and Wilson, R. A. (1998). Murine immune responses to *Schistosoma haematobium* and the vaccine candidate rSh28GST. Parasite Immunology 20(8): 359-367.

Lebens, M., Sun, J. B., Sadeghi, H., Backstrom, M., Olsson, I., Mielcarek, N., Li, B. L., Capron, A., Czerkinsky, C. and Holmgren, J. (2003). A mucosally administered recombinant fusion protein vaccine against schistosomiasis protecting against immunopathology and infection. Vaccine 21(5-6): 514-520.

Lei, J.-h., Liu, W.-q., Sun, C.-s., Tang, C.-l., Li, M.-j., Chen, Y.-l. and Li, Y.-l. (2009). Detection of circulating antigen in serum of mice infected with *Schistosoma japonicum* by immunomagnetic bead ELISA based on IgY. Acta Tropica 111(1): 39-43.

Leigh, R., Ellis, R., Wattlie, J. N., Hirota, J. A., Matthaei, K. I., Foster, P. S., O'Byrne, P. M. and Inman, M. D. (2004). Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. American Journal of Respiratory and Critical Care Medicine 169(7): 860-867.

Leonardi-Bee, J., Pritchard, D., Britton, J. and Collaboration, P. A. (2006). Asthma and current intestinal parasite infection - Systematic review and meta-analysis. American Journal of Respiratory and Critical Care Medicine 174(5): 514-523.

Liang, S. C., Long, A. J., Bennett, F., Whitters, M. J., Karim, R., Collins, M., Goldman, S. J., Dunussi-Joannopoulos, K., Williams, C. M. M., Wright, J. F. and Fouser, L. A. (2007). An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. Journal of Immunology 179(11): 7791-7799.

Liang, Y.-S., Coles, G. C., Doenhoff, M. J. and Southgate, V. R. (2001). *In vitro* responses of praziquantel-resistant and -susceptible *Schistosoma mansoni* to praziquantel. International Journal for Parasitology 31(11): 1227-1235.

Loker, E. S. (1983). A comparative study of the life-histories of mammalian schistosomes. Parasitology 87(Oct): 343-369.

Mabaso, M. L. H., Craig, M., Vounatsou, P. and Smith, T. (2005). Towards empirical description of malaria seasonality in southern Africa: the example of Zimbabwe. Tropical Medicine and International Health 10(9): 909-918.

MacDonald, A. J., Turaga, P. S. D., Harmon-Brown, C., Tierney, T. J., Bennett, K. E., McCarthy, M. C., Simonek, S. C., Enyong, P. A., Moukatte, D. W. and Lustigman, S. (2002). Differential cytokine and antibody responses to adult and larval stages of *Onchocerca volvulus* consistent with the development of concomitant immunity. Infection and Immunity 70(6): 2796-2804.

Maino, V. C. (1998). Rapid assessment of antigen induced cytokine expression in memory T cells by flow cytometry. Veterinary Immunology and Immunopathology 63(1-2): 199-207.

Maizels, R. M., Bundy, D. A. P., Selkirk, M. E., Smith, D. F. and Anderson, R. M. (1993). Immunological modulation and evasion by helminth parasites in human populations. Nature 365(6449): 797-805.

Maizels, R. M., Gomez-Escobar, N., Gregory, W. F., Murray, J. and Zang, X. X. (2001). Immune evasion genes from filarial nematodes. International Journal for Parasitology 31(9): 889-898.

Maizels, R. M. and Yazdanbakhsh, M. (2003). Immune regulation by helminth parasites: cellular and molecular mechanisms. Nature Reviews Immunology 3(9): 733-744.

Maizels, R. M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M. D. and Allen, J. E. (2004). Helminth parasites - masters of regulation. Immunological Reviews 201: 89-116.

Maizels, R. M. (2009). Exploring the immunology of parasitism - from surface antigens to the hygiene hypothesis. Parasitology: 1 -16.

Maizels, R. M., Pearce, E. J., Artis, D., Yazdanbakhsh, M. and Wynn, T. A. (2009). Regulation of pathogenesis and immunity in helminth infections. Journal of Experimental Medicine 206(10): 2059-2066.

Malaquias, L. C. C., Falcao, P. L., Silveira, A. M. S., Gazzinelli, G., Prata, A., Coffman, R. L., Pizziolo, V., Souza, C. P., Colley, D. G. and CorreaOliveira, R. (1997). Cytokine regulation of human immune response to *Schistosoma mansoni*: Analysis of the role of IL-4, IL-5 and IL-10 on peripheral blood mononuclear cell responses. Scandinavian Journal of Immunology 46(4): 393-398.

Mangan, N. E., Fallon, R. E., Smith, P., van Rooijen, N., McKenzie, A. N. and Fallon, P. G. (2004). Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. Journal of Immunology 173(10): 6346-6356.

Mangan, N. E., van Rooijen, N., McKenzie, A. N. J. and Fallon, P. G. (2006). Helminth-modified pulmonary immune response protects mice from allergen-induced airway hyperresponsiveness. Journal of Immunology 176(1): 138-147.

Mannino, D. M., Homa, D. M., Pertowski, C. A., Ashizawa, A., Nixon, L. L., Johnson, C. A., Ball, L. B., Jack, E. and Kang, D. S. (1998). Surveillance for Asthma -- United States, 1960-1995 MMWR CDC Surveillance Summaries 47(SS-1): 1-28.

Marquet, S., Abel, L., Hillaire, D., Dessein, H., Kalil, J., Feingold, J., Weissenbach, J. and Dessein, A. J. (1996). Genetic localization of a locus controlling the intensity of infection by *Schistosoma mansoni* on chromosome 5q31-q33. Nature Genetics 14(2): 181-184.

Marr, N., Hajjar, A. M., Shah, N. R., Novikov, A., Yam, C. S., Caroff, M. and Fernandez, R. C. (2010). Substitution of the *Bordetella pertussis* lipid A phosphate groups with glucosamine is required for robust NF-kappa B activation and release of proinflammatory cytokines in cells expressing human but not murine Toll-Like Receptor 4-MD-2-CD14. Infection and Immunity 78(5): 2060-2069.

Marriner, S. E., Morris, D. L., Dickson, B. and Bogan, J. A. (1986). Pharmacokinetics of albendazole in man. European Journal of Clinical Pharmacology 30(6): 705-708.

Martins-Leite, P., Gazzinelli, G., Alves-Oliveira, L. F., Gazzinelli, A., Malaquias, L. C. C., Correa-Oliveira, R., Teixeira-Carvalho, A. and Silveira, A. M. S. (2008). Effect of chemotherapy with

praziquantel on the production of cytokines and morbidity associated with schistosomiasis mansoni. Antimicrobial Agents and Chemotherapy 52(8): 2780-2786.

Matricardi, P. M., Rosmini, F., Riondino, S., Fortini, M., Ferrigno, L., Rapicetta, M. and Bonini, S. (2000). Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. British Medical Journal 320(7232): 412-417.

Mayringer, I., Reindl, M. and Berger, T. (2000). A critical comparison of frequently used methods for the analysis of tumor necrosis factor-alpha expression by human immune cells. Journal of Immunological Methods 235(1-2): 33-40.

McCune, B. and Grace, J. B. (2002). Analysis of ecological communities, MJM Software Design.

McMahon, J. E. and Kolstrup, N. (1979). Praziquantel: a new schistosomicide against *Schistosoma haematobium*. British Medical Journal 2(6202): 1396-1399.

McManus, D. P., Ross, A. G. P., Sleigh, A. C., Williams, G. M., Yang, W., Li, Y. S., Li, Y., Acosta, L. and Waine, G. J. (1999). Production of interleukin-10 by peripheral blood mononuclear cells from residents of a marshland area in China endemic for *Schistosoma japonicum*. Parasitology International 48(2): 169-177.

McSorley, H. J. and Loukas, A. (2010). The immunology of human hookworm infections. Parasite Immunology 32(8): 549-559.

Mduluza, T., Ndhlovu, P. D., Midzi, N., Mary, C., Paris, C. P., Turner, C. M. R., Chandiwana, S. K., Woolhouse, M. E. J., Dessein, A. J. and Hagan, P. (2001). T cell clones from *Schistosoma haematobium* infected and exposed individuals lacking distinct cytokine profiles for Th1/Th2 polarisation. Memorias Do Instituto Oswaldo Cruz 96: 89-101.

Mduluza, T., Ndhlovu, P. D., Midzi, N., Scott, J. T., Mutapi, F., Mary, C., Couissinier-Paris, P., Turner, C. M. R., Chandiwana, S. K., Woolhouse, M. E. J., Dessein, A. J. and Hagan, P. (2003). Contrasting cellular responses in *Schistosoma haematobium* infected and exposed individuals from areas of high and low transmission in Zimbabwe. Immunology Letters 88(3): 249-256.

Mduluza, T., Ahorlu, C. S., Akoachere, J., Atanga, N. S., Chima, S. C., El-khoby, T., Gasmelseed, N., Mfutso-Bengo, J., Midzi, N., Ndebele, P., Nsimba, S. E. D., Nyika, A. and Vakunseh, B. V. (2007). A gateway to biomedical research in Africa, Nova Science Publishers Inc.

Mduluza, T., Mutapi, F., Ruwona, T., Kaluka, D., Midzi, N. and Ndhlovu, P. D. (2009). Similar cellular responses after treatment with either praziquantel or oxamniquine in *Schistosoma mansoni* infection. Malawi Medical Journal 21(4): 176-182.

Medhat, A., Shehata, M., Bucci, K., Mohamed, S., Dief, A. D. E., Badary, S., Galal, H., Nafeh, M. and King, C. L. (1998). Increased interleukin-4 and interleukin-5 production in response to *Schistosoma haematobium* adult worm antigens correlates with lack of reinfection after treatment. Journal of Infectious Diseases 178(2): 512-519.

Mehta, D. S., Wurster, A. L., Weinmann, A. S. and Grusby, M. J. (2005). NFATc2 and T-bet contribute to T-helper-cell-subset-specific regulation of IL-21 expression. Proceedings of the National Academy of Sciences of the United States of America 102(6): 2016-2021.

Meleney, H. E., Sandground, J. H., Moore, D. V., Most, H. and Carney, B. H. (1953). The histopathology of experimental schistosomiasis: II. Bisexual infections with *S. mansoni, S. japonicum*, and *S. haematobium*. American Journal of Hygiene and Tropical Medicine 2(5): 883-913.

Midzi, N., Sangweme, D., Zinyowera, S., Mapingure, M. P., Brouwer, K. C., Kumar, N., Mutapi, F., Woelk, G. and Mduluza, T. (2008a). Efficacy and side effects of praziquantel treatment against *Schistosoma haematobium* infection among primary school children in Zimbabwe. Transactions of the Royal Society of Tropical Medicine and Hygiene 102(8): 759-766.

Midzi, N., Sangweme, D., Zinyowera, S., Mapingure, M. P., Brouwer, K. C., Munatsi, A., Mutapi, F., Mudzori, J., Kumar, N., Woelk, G. and Mduluza, T. (2008b). The burden of polyparasitism among primary schoolchildren in rural and farming areas in Zimbabwe. Transactions of the Royal Society of Tropical Medicine and Hygiene 102(10): 1039-1045.

Midzi, N., Mtapuri-Zinyowera, S., Mapingure, M. P., Sangweme, D., Chirehwa, M. T., Brouwer, K. C., Mudzori, J., Hlerema, G., Mutapi, F., Kumar, N. and Mduluza, T. (2010). Consequences of polyparasitism on anaemia among primary school children in Zimbabwe. Acta Tropica 115(1-2): 103-111.

Milner, T., Reilly, L., Nausch, N., Midzi, N., Mduluza, T., Maizels, R. M. and Mutapi, F. (2010). Circulating cytokine levels and antibody responses to human *Schistosoma haematobium*: IL-5 and IL-10 levels depend upon age and infection status. Parasite Immunology 32(11-12): 710–721.

Minard, P., Dean, D. A., Jacobson, R. H., Vannier, W. E. and Murrell, K. D. (1978). Immunization of mice with cobalt-60 irradiated *Schistosoma mansoni* cercariae. American Journal of Hygiene and Tropical Medicine 27(1 Pt 1): 76-86.

Minty, A., Chalon, P., Derocq, J. M., Dumont, X., Guillemot, J. C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B. and et al. (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 362(6417): 248-250.

Mitchell, K. M., Mutapi, F. and Woolhouse, M. E. (2008). The predicted impact of immunosuppression upon population age-intensity profiles for schistosomiasis. Parasite Immunology 30(9): 462-470.

Mitchell, K. M. (2010). An analysis of the dynamics of protective immune responses in human populations with endemic schistosome infection. Doctor of Philosophy, The University of Edinburgh.

Mitreva, M., Jasmer, D. P., Zarlenga, D. S., Wang, Z. Y., Abubucker, S., Martin, J., Taylor, C. M., Yin, Y., Fulton, L., Minx, P., Yang, S. P., Warren, W. C., Fulton, R. S., Bhonagiri, V., Zhang, X., Hallsworth-Pepin, K., Clifton, S. W., McCarter, J. P., Appleton, J., Mardis, E. R. and Wilson, R. K. (2011). The draft genome of the parasitic nematode *Trichinella spiralis*. Nature Genetics 43(3): 228-U274.

Mo, H. M., Liu, W. Q., Lei, J. H., Cheng, Y. L., Wang, C. Z. and Li, Y. L. (2007). *Schistosoma japonicum* eggs modulate the activity of CD4(+) CD25(+) Tregs and prevent development of colitis in mice. Experimental Parasitology 116(4): 385-389.

Moingeon, P. (2002). Strategies for designing vaccines eliciting Th1 responses in humans. Journal of Biotechnology 98(2-3): 189-198.

Montenegro, S. M. L., Miranda, P., Mahanty, S., Abath, F. G. C., Teixeira, K. M., Coutinho, E. M., Brinkman, J., Goncalves, I., Domingues, L. A. W., Domingues, A. L. C., Sher, A. and Wynn, T. A. (1999a). Cytokine production in acute versus chronic human schistosomiasis mansoni: The cross-regulatory role of interferon-gamma and interleukin-10 in the responses of peripheral blood mononuclear cells and splenocytes to parasite antigens. Journal of Infectious Diseases 179(6): 1502-1514.

Montenegro, S. N. M., Miranda, P., Mahanty, S., Abath, F. G. C., Teixeira, K. M., Coutinho, E. M., Brinkman, J., Goncalves, I., Domingues, L. A. W., Domingues, A. A. C., Sher, A. and Wynn, T. A. (1999b). Cytokine production in acute versus chronic human schistosomiasis mansoni: the cross-regulatory role of interferon-gamma and interleukin 10 in the responses of peripheral blood mononuclear cells and splenocytes to parasite antigens. The Journal of Infectious Diseases 179(6): 1502-1514.

Montresor, A., Crompton, D. W. T., Bundy, D. A. P., Hall, A. and Savioli, L. (1998). Guidelines for the evaluation of soil-transmitted helminthiasis and schistosomiasis at community level. World Health Organisation Document.

Montresor, A., Crompton, D. W. T., Gyorkos, T. W. and Savioli, L. (2002). Helminth control in school-age children: a guide for managers of control programmes. World Health Organisation Document, World Health Organisation.

Moore-Kucera, J. and Dick, R. P. (2008). Application of 13C-labeled litter and root materials for *in situ* decomposition studies using phospholipid fatty acids. Soil Biology and Biochemistry 40(10): 2485-2493.

Moore, D. V. and Meleney, H. E. (1954). Comparative susceptibility of common laboratory animals to experimental infection with *Schistosoma haematobium*. Journal of Parasitology 40(4): 392-397.

Moore, E. and Doherty, J. F. (2005). Schistosomiasis among travellers returning from Malawi: a common occurrence. Quarterley Journal of Medicine 98(1): 69-70.

Moser, G., Wassom, D. L. and Sher, A. (1980). Studies of the antibody-dependent killing of schistosomula of Schistosoma mansoni employing haptenic target antigens .1. Evidence that the loss in susceptibility to immune damage undergone by developing schistosomula involves a change unrelated to the masking of parasite antigens by host Molecules. Journal of Experimental Medicine 152(1): 41-53.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology 136(7): 2348-2357.

Mosmann, T. R. and Coffman, R. L. (1989). Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology 7: 145-173.

Mosmann, T. R. and Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. Immunology Today 17(3): 138-146.

Mostafa, M. H., Sheweita, S. A. and O'Connor, P. J. (1999). Relationship between schistosomiasis and bladder cancer. Clinical Microbiology Reviews 12(1): 97-111.

Mott, K. E. (1983). A reusable polyamide filter for diagnosis of *S. haematobium* infection by urine filtration. Bulletin de la societe de pathologie exotique et de ses filiales 76(1): 101-104.

Movérare, R., Elfman, L., Björnsson, E. and Stålenheim, G. (2000). Cytokine production by peripheral blood mononuclear cells following birch-pollen immunotherapy. Immunology Letters 73(1): 51-56.

Murrell, K. D., Clark, S., Dean, D. A. and Vannier, W. E. (1979). Influence of mouse strain on induction of resistance with irradiated *Schistosoma mansoni* cercariae. Journal of Parasitology 65(5): 829-831.

Mutapi, F., Ndhlovu, P. D., Hagan, P. and Woolhouse, M. E. J. (1997). A comparison of humoral responses to *Schistosoma haematobium* in areas with low and high levels of infection. Parasite Immunology 19(6): 255-263.

Mutapi, F., Ndhlovu, P. D., Hagan, P., Spicer, J. T., Mduluza, T., Turner, C. M. R., Chandiwana, S. K. and Woolhouse, M. E. J. (1998a). Chemotherapy accelerates the development of acquired immune responses to *Schistosoma haematobium* infection. Journal of Infectious Diseases 178(1): 289-293.

Mutapi, F., Ndhlovu, P. D., Hagan, P. and Woolhouse, M. E. J. (1998b). Changes in specific anti-egg antibody levels following treatment with praziquantel for *Schistosoma haematobium* infection in children. Parasite Immunology 20(12): 595-600.

Mutapi, F., Ndhlovu, P. D., Hagan, P. and Woolhouse, M. E. J. (1999). A comparison of re-infection rates with *Schistosoma haematobium* following chemotherapy in areas with high and low levels of infection. Parasite Immunology 21(5): 253-259.

Mutapi, F., Mduluza, T. and Ndhlovu, P. D. (2002). The effect of treatment on the age-antibody relationship in children infected with *Schistosoma mansoni* and *Schistosoma haematobium*. Memorias do Instituto Oswaldo Cruz 97: 173-180.

Mutapi, F. and Roddam, A. (2002). p values for pathogens: statistical inference from infectious-disease data. Lancet Infectious Diseases 2(4): 219-230.

Mutapi, F., Hagan, P., Woolhouse, M. E. J., Mduluza, T. and Ndhlovu, P. D. (2003). Chemotherapy-induced, age-related changes in antischistosome antibody responses. Parasite Immunology 25(2): 87-97.

Mutapi, F., Burchmore, R., Mduluza, T., Foucher, A., Harcus, Y., Nicoll, G., Midzi, N., Turner, C. M. and Maizels, R. M. (2005). Praziquantel treatment of individuals exposed to *Schistosoma haematobium* enhances serological recognition of defined parasite antigens. Journal of Infectious Diseases 192(6): 1108-1118.

Mutapi, F., Mduluza, T., Gomez-Escobar, N., Gregory, W. F., Fernandez, C., Midzi, N. and Maizels, R. M. (2006). Immuno-epidemiology of human *Schistosoma haematobium* infection: preferential IgG3 antibody responsiveness to a recombinant antigen dependent on age and parasite burden. BMC Infectious Diseases 6.

Mutapi, F., Roussilhon, C., Mduluza, T. and Druilhe, P. (2007a). Anti-malaria humoral responses in children exposed to *Plasmodium falciparum* and *Schistosoma haematobium*. Memorias do Instituto Oswaldo Cruz 102(3): 405-409.

Mutapi, F., Winborn, G., Midzi, N., Taylor, M., Mduluza, T. and Maizels, R. M. (2007b). Cytokine responses to *Schistosoma haematobium* in a Zimbabwean population: contrasting profiles for IFN-gamma, IL-4, IL-5 and IL-10 with age. BMC Infectious Diseases 7(139).

Mutapi, F., Burchmore, R., Mduluza, T., Midzi, N., Turner, C. M. R. and Maizels, R. M. (2008). Age-related and infection intensity-related shifts in antibody recognition of defined protein antigens in a schistosome-exposed population. Journal of Infectious Diseases 198(2): 167-175.

Mutapi, F., Bourke, C., Harcus, Y., Midzi, N., Mduluza, T., Turner, C. M., Burchmore, R. and Maizels, R. M. (2011a). Differential recognition patterns of *Schistosoma haematobium* adult worm antigens by the human antibodies IgA, IgE, IgG1 and IgG4. Parasite Immunology 33(3): 181-192.

Mutapi, F., Imai, N., Nausch, N., Bourke, C. D., Rujeni, N., Mitchell, K. M., Midzi, N., Woolhouse, M. E. J., Maizels, R. M. and Mduluza, T. (2011b). Schistosome infection intensity is inversely related to auto-reactive antibody levels. PLoS ONE 6(5): e19149.

Mutapi, F., Rujeni, N., Bourke, C., Mitchell, K., Appleby, L., Nausch, N., Midzi, N. and Mduluza, T. (2011c). *Schistosoma haematobium* treatment in 1-5 Year old children: Safety and efficacy of the antihelminthic drug praziquantel. PLoS Neglected Tropical Diseases 5(5): e1143.

Na-Bangchang, K., Kietinun, S., Pawa, K. K., Hanpitakpong, W., Na-Bangchang, C. and Lazdins, J. (2006). Assessments of pharmacokinetic drug interactions and tolerability of albendazole, praziquantel and ivermectin combinations. Transactions of the Royal Society of Tropical Medicine and Hygiene 100(4): 335-345.

Nanduri, J., Dennis, J. E., Rosenberry, T. L., Mahmoud, A. A. F. and Tartakoff, A. M. (1991). Glycocalyx of bodies versus tails of *Schistosoma mansoni* cercariae lectin-binding, size, charge, and electron-microscopic characterization. Journal of Biological Chemistry 266(2): 1341-1347.

Nathan, C. F., Prendergast, T. J., Wiebe, M. E., Stanley, E. R., Platzer, E., Remold, H. G., Welte, K., Rubin, B. Y. and Murray, H. W. (1984). Activation of human macrophages. Comparison of other cytokines with interferon-gamma. Journal of Experimental Medicine 160(2): 600-605.

Naus, C. W. A., Jones, F. M., Satti, M. Z., Joseph, S., Riley, E. M., Kimani, G., Mwatha, J. K., Kariuki, C. H., Ouma, J. H., Kabatereine, N. B., Vennervald, B. J. and Dunne, D. W. (2003). Serological responses among individuals in areas where both schistosomiasis and malaria are endemic: Cross-reactivity between *Schistosoma mansoni* and *Plasmodium falciparum*. Journal of Infectious Diseases 187(8): 1272-1282.

Nausch, N., Midzi, N., Mduluza, T., Maizels, R. M. and Mutapi, F. (2011). Regulatory and activated T cells in human *Schistosoma haematobium* infections. PLoS ONE 6(2): e16860.

Nibbeling, H. A. M., VanEtten, L., Fillie, Y. E. and Deelder, A. M. (1997). Enhanced detection of *Schistosoma* circulating antigens by testing 1 ml urine samples using immunomagnetic beads. Acta Tropica 66(2): 85-92.

Nibbeling, H. A. M., Van Lieshout, L. and Deelder, A. M. (1998). Levels of circulating soluble egg antigen in urine of individuals infected with *Schistosoma mansoni* before and after treatment with praziquantel. Transactions of the Royal Society of Tropical Medicine and Hygiene 92(6): 675-677.

NIH. (2009). Efficacy of vaccine Sh28GST in association with praziquantel (PZQ) for prevention of clinical recurrences of *Schistosoma haematobium* pathology (Bilhvax). Retrieved 6th June, 2011, from http://clinicaltrials.gov/show/NCT00870649.

Njomo, D. W., Tomono, N., Muhoho, N., Mitsui, Y., Josyline, J. C. and Mwandawro, C. S. (2010). The adverse effects of albendazole and praziquantel in mass drug administration by trained schoolteachers. African Journal of Health Sciences 17(3 - 4).

O'Leary, S., O'Sullivan, M. P. and Keane, J. (2010). IL-10 blocks phagosome maturation in *Mycobacterium tuberculosis*-infected human macrophages. American Journal of Respiratory Cell and Molecular Biology.

Okano, M., Satoskar, A. R., Nishizaki, K. and Harn, D. A. (2001). Lacto-N-fucopentaose III found on *Schitosoma mansoni* egg antigens functions as adjuvant for proteins by inducing Th2-type response. Journal of Immunology 167(1): 442-450.

Olds, G. R., King, C., Hewlett, J., Olveda, R., Wu, G., Ouma, J., Peters, P., McGarvey, S., Odhiambo, O., Koech, D., Liu, C. Y., Aligui, G., Gachihi, G., Kombe, Y., Parraga, I., Ramirez, B., Whalen, C., Horton, R. J. and Reeve, P. (1999). Double-blind placebo-controlled study of concurrent administration of albendazole and praziquantel in schoolchildren with schistosomiasis and geohelminths. Journal of Infectious Diseases 179(4): 996-1003.

Opara, K. N., Udoidung, N. I. and Ukpong, I. G. (2007). Genitourinary schistosomiasis among pre-primary schoolchildren in a rural community within the Cross river basin, Nigeria. Journal of Helminthology 81(4): 393-397.

Oppenheim, J. J. (2001). Cytokines: Past, present, and future. International Journal of Haematology 74(1): 3-8.

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y. M., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M. R., Gorman, D., Wagner, J., Zurawski, S., Liu, Y. J., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F. and Kastelein, R. A. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13(5): 715-725.

Osakwe, C. E., Bleotu, C., Chifiriuc, M. C., Grancea, C., Otelea, D., Paraschiv, S., Petrea, S., Dinu, M., Baicus, C., Streinu-Cercel, A. and Lazar, V. (2010). TH1/TH2 cytokine levels as an indicator for disease progression in human immunodeficiency virus type 1 infection and response to antiretroviral therapy. Roumanian Archives of Microbiology and Immunology 69(1): 24-34.

Osborne, J. W. (2002). Notes on the use of data transformation. Practical Assessment, Research and Evaluation 8(6).

Ottesen, E. A., Hiatt, R. A., Cheever, A. W., Sotomayor, Z. R. and Neva, F. A. (1978). Acquisition and loss of antigen-specific cellular immune responsiveness in acute and chronic schistosomiasis in man. Clinical and Experimental Immunology 33(1): 38-47.

Pahwa, R., Jaggaiahgari, S., Pahwa, S., Inverardi, L., Tzakis, A. and Ricordi, C. (2010). Isolation and expansion of human natural T regulatory cells for cellular therapy. Journal of Immunology Methods 363(1): 67-79.

Pastrana, D. V., Raghavan, N., FitzGerald, P., Eisinger, S. W., Metz, C., Bucala, R., Schleimer, R. P., Bickel, C. and Scott, A. L. (1998). Filarial nematode parasites secrete a homologue of the human cytokine macrophage migration inhibitory factor. Infection and Immunity 66(12): 5955-5963.

Paveley, R. A., Aynsley, S. A., Cook, P. C., Turner, J. D. and Mountford, A. P. (2009). Fluorescent imaging of antigen released by a skin-invading helminth reveals differential uptake and activation profiles by antigen presenting cells. PLoS Neglected Tropical Diseases 3(10): e528.

Pearce, E. J., Caspar, P., Grzych, J. M., Lewis, F. A. and Sher, A. (1991). Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. Journal of Experimental Medicine 173(1): 159-166.

Pearce, E. J. and MacDonald, A. S. (2002). The immunobiology of schistosomiasis. Nature Reviews Immunology 2(7): 499-511.

Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. and Dahinden, C. A. (2009). Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. Blood 113(7): 1526-1534.

Pengsaa, K., Na-Bangchang, K., Limkittikul, K., Kabkaew, K., Lapphra, K., Sirivichayakul, C., Wisetsing, P., Pojjaroen-Anant, C., Chanthavanich, P. and Subchareon, A. (2004). Pharmacokinetic investigation of albendazole and praziquantel in Thai children infected with *Giardia intestinalis*. Annals of Tropical Medicine and Parasitology 98: 349-357.

Perona-Wright, G., Mohrs, K., Mayer, K. D. and Mohrs, M. (2010). Differential regulation of IL-4Rα expression by antigen versus cytokine stimulation characterizes Th2 progression *in vivo*. Journal of Immunology 184(2): 615-623.

Pesce, J., Kaviratne, M., Ramalingam, T. R., Thompson, R. W., Urban, J. F., Cheever, A. W., Young, D. A., Collins, M., Grusby, M. J. and Wynn, T. A. (2006). The IL-21 receptor augments Th2 effector function and alternative macrophage activation. Journal of Clinical Investigation 116(7): 2044-2055.

Pesce, J. T., Ramalingam, T. R., Mentink-Kane, M. M., Wilson, M. S., El Kasmi, K. C., Smith, A. M., Thompson, R. W., Cheever, A. W., Murray, P. J. and Wynn, T. A. (2009). Arginase-1–expressing macrophages suppress Th2 cytokine–driven inflammation and fibrosis. PLoS Pathogens 5(4): e1000371.

Picquet, M., Vercruysse, J., Shaw, D. J., Diop, M. and Ly, A. (1998). Efficacy of praziquantel against Schistosoma mansoni in northern Senegal. Transactions of the Royal Society of Tropical Medicine and Hygiene 92(1): 90-93.

Pierce, R. J., Balloul, J. M., Grzych, J. M., Dissous, C., Auriault, C., Boulanger, D., Capron, M., Sondermeyer, P., Lecocq, J. P. and Capron, A. (1987). GP38, P28-I and P28-II: candidates for a vaccine against schistosomiasis. Memorias de Instituto Oswaldo Cruz 82 Suppl 4: 111-114.

Platt, T. R. and Brooks, D. R. (1997). Evolution of the schistosomes (Digenea: Schistosomatoidea): The origin of dioecy and colonization of the venous system. Journal of Parasitology 83(6): 1035-1044.

Polman, K., Van Lieshout, L., Gryseels, B. and Deelder, A. M. (1998). Age-related worm load and worm fecundity patterns in human populations, as indicated by schistosome circulating antigens. Memorias Do Instituto Oswaldo Cruz 93 Suppl 1: 123-125.

Polman, K., Diakhate, M. M., Engels, D., Nahimana, S., Van Dam, G. J., Ferreira, S. T. M. F., Deelder, A. M. and Gryseels, B. (2000). Specificity of circulating antigen detection for schistosomiasis mansoni in Senegal and Burundi. Tropical Medicine and International Health 5(8): 534-537.

Polman, K., Stelma, F. F., De Vlas, S. J., Sow, S., Fathers, L., Le Cessie, S., Talla, I., Deelder, A. M. and Gryseels, B. (2001). Dynamics of egg counts and circulating antigen levels in a recent Schistosoma mansoni focus in northern Senegal. Tropical Medicine and International Health 6(7): 538-544.

Quinnell, R. J., Woolhouse, M. E. J., Walsh, E. A. and Pritchard, D. I. (1995). Immunoepidemiology of human necatoriasis: correlations between antibody responses and parasite burdens. Parasite Immunology 17(6): 313-318.

Quinnell, R. J. (2003). Genetics of susceptibility to human helminth infection. International Journal for Parasitology 33(11): 1219-1231.

Quinnell, R. J., Bethony, J. and Pritchard, D. I. (2004a). The immunoepidemiology of human hookworm infection. Parasite Immunology 26(11-12): 443-454.

Quinnell, R. J., Pritchard, D. I., Raiko, A., Brown, A. P. and Shaw, M. A. (2004b). Immune responses in human necatoriasis: Association between interleukin-5 responses and resistance to reinfection. Journal of Infectious Diseases 190(3): 430-438.

Rahoud, S. A., Mergani, A., Khamis, A. H., Saeed, O. K., Mohamed-Ali, Q., Dessein, A. J. and Elwali, N. E. M. A. (2010). Factors controlling the effect of praziquantel on liver fibrosis in *Schistosoma mansoni*-infected patients. FEMS Immunology and Medical Microbiology 58(1): 106-112.

Ramaswamy, K., Kumar, P. and He, Y. X. (2000). A role for parasite-induced PGE2 in IL-10-mediated host immunoregulation by skin stage schistosomula of *Schistosoma mansoni*. Journal of Immunology 165(8): 4567-4574.

Rao, A. and Avni, O. (2000). Molecular aspects of T-cell differentiation. British Medical Bulletin 56(4): 969-984.

Raso, G., Luginbuhl, A., Adjoua, C. A., Tian-Bi, N. T., Silue, K. D., Matthys, B., Vounatsou, P., Wang, Y., Dumas, M. E., Holmes, E., Singer, B. H., Tanner, M., N'Goran, E. K. and Utzinger, J. (2004). Multiple parasite infections and their relationship to self-reported morbidity in a community of rural Cote d'Ivoire. International Journal of Epidemiology 33(5): 1092-1102.

Reddy, A. and Fried, B. (2007). The use of *Trichuris suis* and other helminth therapies to treat Crohn's disease. Parasitology Research 100(5): 921-927.

Redman, C. A., Robertson, A., Fallon, P. G., Modha, J., Kusel, J. R., Doenhoff, M. J. and Martin, R. J. (1996). Praziquantel: An urgent and exciting challenge. Parasitology Today 12(1): 14-20.

Reilly, L., Magkrioti, C., Mduluza, T., Cavanagh, D. R. and Mutapi, F. (2008). Effect of treating *Schistosoma haematobium* infection on *Plasmodium falciparum*-specific antibody responses. BMC Infectious Diseases 8(158).

Reimert, C. A., Fitzsimmons, C. M., Joseph, S., Mwatha, J. K., Jones, F. M., Kimani, G., Hoffmann, K. F., Booth, M., Kabatereine, N. B., Dunne, D. W. and Vennervald, B. J. (2006). Eosinophil activity in *Schistosoma mansoni* infections *in vivo* and *in vitro* in relation to plasma cytokine profile pre- and posttreatment with praziquantel. Clinical and Vaccine Immunology 13(5): 584-593.

Remick, D. G., Newcomb, D. E. and Friedland, J. S. (2000). Whole blood assays for cytokine production. Methods in molecular medicine. T. J. Evans. Totowa, Humana Press Inc. 36**:** 101-112.

Remoué, F., Rogerie, F., Gallissot, M. C., Guyatt, Helen L., Neyrinck, J. L., Diakkhate, M., Niang, M., Butterworth, A., Auriault, C., Capron, A. and Riveau, G. (2000). Sex dependent neutralizing humoral response to *Schistosoma mansoni* 28GST antigen in infected human populations. Journal of Infectious Diseases 181(5): 1855-1859.

Remoué, F., To Van, D., Schacht, A. M., Picquet, M., Garraud, O., Vercruysse, J., Ly, A., Capron, A. and Riveau, G. (2001). Gender-dependent specific immune response during chronic human schistosomiasis haematobium. Clinical and Experimental Immunology 124(1): 62-68.

Rice, W. R. (1989). Analyzing tables of statistical tests. Evolution 43(1): 223-225.

Richter, D. and Harn, D. A. (1993). Candidate vaccine antigens identified by antibodies from mice vaccinated with 15- or 50-kilorad-irradiated cercariae of *Schistosoma mansoni*. Infection and Immunity 61(1): 146-154.

Riedler, Eder, Oberfeld and Schreuer (2000). Austrian children living on a farm have less hay fever, asthma and allergic sensitization. Clinical and Experimental Allergy 30(2): 194-200.

Rihet, P., Demeure, C. E., Bourgois, A., Prata, A. and Dessein, A. J. (1991). Evidence for an association between human resistance to Schistosoma mansoni and high anti-larval IgE levels. European Journal of Immunology 21(11): 2679-2686.

Rivino, L., Gruarin, P., Haringer, B., Steinfelder, S., Lozza, L., Steckel, B., Weick, A., Sugliano, E., Jarrossay, D., Kuhl, A. A., Loddenkemper, C., Abrignani, S., Sallusto, F., Lanzavecchia, A. and Geginat, J. (2010). CCR6 is expressed on an IL-10-producing, autoreactive memory T cell population with context-dependent regulatory function. Journal of Experimental Medicine 207(3): 565-577.

Roberts, M., Butterworth, A. E., Kimani, G., Kamau, T., Fulford, A. J., Dunne, D. W., Ouma, J. H. and Sturrock, R. F. (1993). Immunity after treatment of human schistosomiasis: association between cellular responses and resistance to reinfection. Infection and Immunity 61(12): 4984-4993.

Roberts, S. M., Wilson, R. A., Ouma, J. H., Kariuki, H. C., Koech, D., Siongok, T. K. A., Sturrock, R. F. and Butterworth, A. E. (1987). Immunity after treatment of human schistosomiasis mansoni: quantitative and qualitative antibody responses to tegumental membrane antigens prepared from adult worms. Transactions of the Royal Society of Tropical Medicine and Hygiene 81(5): 786-793.

Rocha, B., Dautigny, N. and Pereira, P. (1989). Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. European Journal of Immunology 19(5): 905-911.

Rudge, J. W., Stothard, J. R., Basanez, M. G., Mgeni, A. F., Khamis, I. S., Khamis, A. N. and Rollinson, D. (2008). Micro-epidemiology of urinary schistosomiasis in Zanzibar: Local risk factors associated with distribution of infections among schoolchildren and relevance for control. Acta Tropica 105(1): 45-54.

Rummel, R. J. (1970). Applied Factor Analysis, Northwestern University Press.

Rutitzky, L. I., Bazzone, L., Shainheit, M. G., Joyce-Shaikh, B., Cua, D. J. and Stadecker, M. J. (2008). IL-23 is required for the development of severe egg-induced immunopathology in schistosomiasis and for lesional expression of IL-17. Journal of Immunology 180(4): 2486-2495.

Rutitzky, L. I., Smith, P. M. and Stadecker, M. J. (2009). T-bet protects against exacerbation of schistosome egg-induced immunopathology by regulating Th17-mediated inflammation. European Journal of Immunology 39(9): 2470-2481.

Saathoff, E., Olsen, A., Magnussen, P., Kvalsvig, J. D., Becker, W. and Appleton, C. C. (2004). Patterns of *Schistosoma haematobium* infection, impact of praziquantel treatment and re-infection after treatment in a cohort of schoolchildren from rural KwaZulu-Natal/South Africa. BMC Infectious Diseases 4: -.

Sabah, A. A., Fletcher, C., Webbe, G. and Doenhoff, M. J. (1986). *Schistosoma mansoni*: chemotherapy of infections of different ages. Experimental Parasitology 61(3): 294-303.

Saito, H., Howie, K., Wattie, J., Denburg, A., Ellis, R., Inman, M. D. and Denburg, J. A. (2001). Allergen-induced murine upper airway inflammation: local and systemic changes in murine experimental allergic rhinitis. Immunology 104(2): 226-234.

Salter, J. P., Choe, Y., Albrecht, H., Franklin, C., Lim, K. C., Craik, C. S. and McKerrow, J. H. (2002). Cercarial elastase is encoded by a functionally conserved gene family across multiple species of schistosomes. Journal of Biological Chemistry 277(27): 24618-24624.

Samuelson, J. C. and Caulfield, J. P. (1985). The cercarial glycocalyx of *Schistosoma mansoni*. Journal of Cell Biology 100(5): 1423-1434.

Sanderson, C. (1992). Interleukin-5, eosinophils and disease. Blood 79(12): 3101-3109.

Sartono, E., Kruize, Y. C. M., Kurniawan, A., Maizels, R. M. and Yazdanbakhsh, M. (1997). Depression of antigen-specific interleukin-5 and interferon-gamma responses in human lymphatic filariasis as a function of clinical status and age. Journal of Infectious Diseases 175(5): 1276-1280.

Satti, M. Z., Lind, P., Vennervald, B. J., Sulaiman, S. M., Daffalla, A. A. and Ghalib, H. W. (1996). Specific immunoglobulin measurements related to exposure and resistance to *Schistosoma mansoni* infection in Sudanese canal cleaners. Clinical and Experimental Immunology 106(1): 45-54.

Scavuzzo, M. C., Rocchi, V., Fattori, B., Ambrogi, F., Carpi, A., Ruffoli, R., Manganelli, S. and Giannessi, F. (2003). Cytokine secretion in nasal mucus of normal subjects and patients with allergic rhinitis. Biomedecine and Pharmacotherapy 57(8): 366-371.

Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S. and Dinarello, C. (1990). Correlations and interactions in the production of interleukin-6 (IL- 6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood 75(1): 40-47.

Schmitz, J., Owyang, A., Oldham, E., Song, Y. L., Murphy, E., McClanahan, T. K., Zurawski, G., Moshrefi, M., Qin, J. Z., Li, X. X., Gorman, D. M., Bazan, J. F. and Kastelein, R. A. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 23(5): 479-490.

Schnyder, B., Lugli, S., Feng, N. P., Etter, H., Lutz, R. A., Ryffel, B., Sugamura, K., WunderliAllenspach, H. and Moser, R. (1996). Interleukin-4 (IL-4) and IL-13 bind to a shared heterodimeric complex on endothelial cells mediating vascular cell adhesion molecule-1 induction in the absence of the common gamma chain. Blood 87(10): 4286-4295.

Schramm, G., Falcone, F. H., Gronow, A., Haisch, K., Mamat, U., Doenhoff, M. J., Oliveira, G., Galle, J., Dahinden, C. A. and Haas, H. (2003). Molecular characterization of an interleukin-4-inducing factor from *Schistosoma mansoni* eggs. Journal of Biological Chemistry 278(20): 18384-18392.

Scott, J. T., Turner, C. M. R., Mutapi, F., Woolhouse, M. E. J., Chandiwana, S. K., Mduluza, T., Ndhlovu, P. D. and Hagan, P. (2000). Dissociation of interleukin-4 and interleukin-5 production following treatment for *Schistosoma haematobium* infection in humans. Parasite Immunology 22(7): 341-348.

Scott, J. T., Turner, C. M. R., Mutapi, F., Woolhouse, M. E. J., Ndhlovu, P. D. and Hagan, P. (2001). Cytokine responses to mitogen and *Schistosoma haematobium* antigens are different in children with distinct infection histories. Parasite Immunology 23(10): 519-526.

Senti, G., Johansen, P., Gomez, J. M., Varicka, B. M. P. and Kundig, T. M. (2005). Efficacy and safety of allergen-specific immunotherapy in rhinitis, rhinoconjunctivitis, and bee/wasp venom allergies. International Reviews of Immunology 24(5-6): 519-531.

Senti, G., Vavricka, B. M. P., Erdmann, I., Diaz, M. I., Markus, R., McCormack, S. J., Simard, J. J., Wuthrich, B., Crameri, R., Graf, N., Johansen, P. and Kundig, T. M. (2008). Intralymphatic allergen administration renders specific immunotherapy faster and safer: A randomized controlled trial. Proceedings of the National Academy of Sciences of the United States of America 105(46): 17908-17912.

Shainheit, M. G., Smith, P. M., Bazzone, L. E., Wang, A. C., Rutitzky, L. I. and Stadecker, M. J. (2008). Dendritic cell IL-23 and IL-1 production in response to schistosome eggs induces Th17 cells in a mouse strain prone to severe immunopathology. Journal of Immunology 181(12): 8559-8567.

Shaw, M. K. (1990). *Schistosoma mansoni:* stage-dependent damage after *in vivo* treatment with praziquantel. Parasitology 100 Pt 1: 65-72.

Shuhua, X., Binggui, S., Chollet, J. and Tanner, M. (2000). Tegumental changes in adult Schistosoma mansoni harboured in mice treated with praziquantel enantiomers. Acta Tropica 76(2): 107-117.

Silveira-Lemos, D., Teixeira-Carvalho, A., Martins-Filho, O. A., Alves Oliveira, L. F., Costa-Silva, M. F., Matoso, L. F., de Souza, L. J., Gazzinelli, A. and Corrêa-Oliveira, R. (2008). Eosinophil activation status, cytokines and liver fibrosis in *Schistosoma mansoni* infected patients. Acta Tropica 108(2-3): 150-159.

Silveira, A. M. S., Gazzinelli, G., Alves-Oliveira, L. F., Bethony, J., Gazzinelli, A., Carvalho-Queiroz, C., Alvarez, M. C. B., Lima-Silva, F. C., Prata, A., LoVerde, P. T. and Correa-Oliveira, R. (2004). Human schistosomiasis mansoni: Intensity of infection differentially affects the production of interleukin-10, interferon-gamma and interleukin-13 by soluble egg antigen or adult worm

antigen stimulated cultures. Transactions of the Royal Society of Tropical Medicine and Hygiene 98(9): 514-519.

Sirivichayakul, C., Pojjaroen-anant, C., Wisetsing, P., Chanthavanich, P., Praevanit, R., Limkittikul, K. and Pengsaa, K. (2001). A comparative trial of albendazole alone versus combination of albendazole and praziquantel for treatment of *Trichuris trichiura* infection. Southeast Asian Journal of Tropical Medicine and Public Health 32(2): 297-301.

Sissoko, M. S., Dabo, A., Traore, H., Diallo, M., Traore, B., Konate, D., Niare, B., Diakite, M., Kamate, B., Traore, A., Bathily, A., Tapily, A., Toure, O. B., Cauwenbergh, S., Jansen, H. F. and Doumbo, O. K. (2009). Efficacy of artesunate + sulfamethoxypyrazine/pyrimethamine versus praziquantel in the treatment of *Schistosoma haematobium* in children. PLoS ONE 4(10): e6732.

Smith, B. J., Davies, P. E. and Munks, S. A. (2009). Changes in benthic macroinvertebrate communities in upper catchment streams across a gradient of catchment forest operation history. Forest Ecology and Management 257(10): 2166-2174.

Smith, P., Walsh, C. M., Mangan, N. E., Fallon, R. E., Sayers, J. R., McKenzie, A. N. J. and Fallon, P. G. (2004). *Schistosoma mansoni* worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. Journal of Immunology 173(2): 1240-1248.

Smithers, S. R. and Terry, R. J. (1967). Resistance to experimental infection with *Schistosoma mansoni* in rhesus monkeys induced by the transfer of adult worms. Transactions of the Royal Society of Tropical Medicine and Hygiene 61(4): 517-533.

Smits, H. H., Everts, B., Hartgers, F. C. and Yazdanbakhsh, M. (2010). Chronic helminth infections protect against allergic diseases by active regulatory processes. Current Allergy and Asthma Reports 10(1): 3-12.

Sokal, R. R. and Rohlf, F. J. (1995a). Chapter 16: multiple and curvilinear regression. Biometry: Third Edition, W.H.Freeman & Co Ltd**:** 609-684.

Sokal, R. R. and Rohlf, F. J. (1995b). Chapter 9: single-classification analysis of variance. Biometry: Third Edition, W.H.Freeman & Co Ltd**:** 609-684.

Sokal, R. R. and Rohlf, F. J. (1995c). Chapter 13: assumptions of analysis of variance. Biometry: Third Edition, W.H.Freeman & Co Ltd**:** 609-684.

Sokal, R. R. and Rohlf, F. J. (1995d). Chapter 17:analysis of frequencies. Biometry: Third Edition, W.H.Freeman & Co Ltd**:** 685-793.

Soonawala, D., Geerts, J.-W. H. J., de Mos, M., Yazdanbakhsh, M. and Visser, L. G. (2011). The immune response to schistosome antigens in formerly infected travelers. American Journal of Tropical Medicine and Hygiene 84(1): 43-47.

Standley, C. J., Lwambo, N. J. S., Lange, C. N., Kariuki, H. C., Adriko, M. and Stothard, J. R. (2010). Performance of circulating cathodic antigen (CCA) urine-dipsticks for rapid detection of intestinal schistosomiasis in schoolchildren from shoreline communities of Lake Victoria. Parasites and Vectors 3: -.

Stanworth, D. R. and Smith, A. K. (1973). Inhibition of reagin-mediated PCA reactions in baboons by the human IgG4 sub-class. Clinical and Experimental Allergy 3(1): 37-41.

Steenhard, N. R., Kringel, H., Roepstorff, A., Thamsborg, S. M. and Jungersen, G. (2007). Parasite-specific IL-4 responses in *Ascaris suum* and *Trichuris suis*-infected pigs evaluated by ELISPOT. Parasite Immunology 29(10): 535-538.

Steinfelder, S., Andersen, J. F., Cannons, J. L., Feng, C. G., Joshi, M., Dwyer, D., Caspar, P., Schwartzberg, P. L., Sher, A. and Jankovic, D. (2009). The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). Journal of Experimental Medicine 206(8): 1681-1690.

Stirewalt, M. A. and Kruidenier, F. J. (1961). Activity of the acetabular secretory apparatus of cercariae of *Schistosoma mansoni* under experimental conditions. Experimental Parasitology 11(2-3): 191-198, IN193, 199-211.

Stoll, N. R. (1947). This wormy world. Journal of Parasitology 33(1): 1-18.

Stothard, J. R., Sousa-Figuereido, J. C., Betson, M., Adriko, M., Arinaitwe, M., Rowell, C., Besiyge, F. and Kabatereine, N. B. (2011). *Schistosoma mansoni* infections in young children: When are schistosome antigens in urine, eggs in stool and antibodies to eggs first detectable? PLoS Neglected Tropical Diseases 5(1): e938.

Strengell, M., Sareneva, T., Foster, D., Julkunen, I. and Matikainen, S. (2002). IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. Journal of Immunology 169(7): 3600-3605.

Summers, R. W., Elliott, D. E., Qadir, K., Urban, J. F., Thompson, R. and Weinstock, J. V. (2003). *Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory bowel disease. American Journal of Gastroenterology 98(9): 2034-2041.

Summers, R. W., Elliott, D. E., Urban, J. F., Thompson, R. and Weinstock, J. V. (2005a). *Trichuris suis* therapy in Crohn's disease. Gut 54(1): 87-90.

Summers, R. W., Elliott, D. E., Urban, J. F., Thompson, R. A. and Weinstock, J. V. (2005b). *Trichuris suis* therapy for active ulcerative colitis: A randomized controlled trial. Gastroenterology 128(4): 825-832.

Summers, R. W., Elliott, D. E. and Weinstock, J. V. (2005c). Reply. Gastroenterology 129(2): 769-769.

Summers, R. W., Elliott, D. E. and Weinstock, J. V. (2010). *Trichuris suis* might be effective in treating allergic rhinitis. Journal of Allergy and Clinical Immunology 125(3): 766-767.

Suni, M. A., Picker, L. J. and Maino, V. C. (1998). Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. Journal of Immunological Methods 212(1): 89-98.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H. (2000). A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. Cell 100(6): 655-669.

Tarafder, M. R., Carabin, H., Joseph, L., Balolong Jr, E., Olveda, R. and McGarvey, S. T. (2010). Estimating the sensitivity and specificity of Kato-Katz stool examination technique for detection of hookworms, *Ascaris lumbricoides* and *Trichuris trichiura* infections in humans in the absence of a 'gold standard'. International Journal for Parasitology 40(4): 399-404.

Taylor, J. B., Vidal, A., Torpier, G., Meyer, D. J., Roitsch, C., Balloul, J. M., Southan, C., Sondermeyer, P., Pemble, S., Lecocq, J. P. and et al. (1988). The glutathione transferase activity and tissue distribution of a cloned Mr28K protective antigen of *Schistosoma mansoni*. EMBO Journal 7(2): 465-472.

Taylor, P. and Mutambu, S. L. (1986). A review of the malaria situation in Zimbabwe with special reference to the period 1972-1981. Transactions of the Royal Society of Tropical Medicine and Hygiene 80(1): 12-19.

Tchuem Tchuente, L. A., Shaw, D. J., Polla, L., Cioli, D. and Vercruysse, J. (2004). Efficacy of praziquantel against *Schistosoma haematobium* infection in children American Journal of Tropical Medicine Hygeine 71(6): 778-782.

Thomas, P. G., Carter, M. R., Atochina, O., Da'Dara, A. A., Piskorska, D., McGuire, E. and Harn, D. A. (2003). Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. Journal of Immunology 171(11): 5837-5841.

Tingley, G. A., Butterworth, A. E., Anderson, R. M., Kariuki, H. C., Koech, D., Mugambi, M., Ouma, J. H., Siongok, T. K. A. and Sturrock, R. F. (1988). Predisposition of humans to infection with *Schistosoma mansoni*: evidence from the reinfection of individuals following chemotherapy. Transactions of the Royal Society of Tropical Medicine and Hygiene 82(3): 448-452.

Todd, C. W., Goodgame, R. W. and Colley, D. G. (1979). Immune responses during human schistosomiasis mansoni: V. Suppression of schistosome antigen-specific lymphocyte blastogenesis by adherent/phagocytic cells. Journal of Immunology 122(4): 1440-1446.

Tosato, G., Gerrard, T. L., Goldman, N. G. and Pike, S. E. (1988). Stimulation of EBV-activated human B cells by monocytes and monocyte products. Role of IFN-beta 2/B cell stimulatory factor 2/IL-6. Journal of Immunology 140(12): 4329-4336.

Tosato, G. and Pike, S. E. (1988). Interferon-beta 2/interleukin 6 is a co-stimulant for human T lymphocytes. Journal of Immunology 141(5): 1556-1562.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nature Reviews Immunology 3(2): 133-146.

Trottein, F., Godin, C., Pierce, R. J., Sellin, B., Taylor, M. G., Gorillot, I., Silva, M. S., Lecocq, J.-P. and Capron, A. (1992a). Inter-species variation of schistosome 28-kDa glutathione S-transferases. Molecular and Biochemical Parasitology 54(1): 63-72.

Trottein, F., Vaney, M. C., Bachet, B., Pierce, R. J., Colloch, N., Lecocq, J. P., Capron, A. and Mornon, J. P. (1992b). Crystallization and preliminary-X-ray diffraction studies of a protective cloned 28 Kda glutathione-S-transferase from *Schistosoma mansoni*. Journal of Molecular Biology 224(2): 515-518.

Trujillo-Vargas, C. M., Werner-Klein, M., Wohlleben, G., Polte, T., Hansen, G., Ehlers, S. and Erb, K. J. (2007). Helminth-derived products inhibit the development of allergic responses in mice. American Journal of Respiritory and Critical Care Medicine 175(4): 336-344.

Turcotte, I., Quideau, S. A. and Oh, S.-W. (2009). Organic matter quality in reclaimed boreal forest soils following oil sands mining. Organic Geochemistry 40(4): 510-519.

Turnbull, A. V. and Rivier, C. L. (1999). Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: Actions and mechanisms of action. Physiological Reviews 79(1): 1-71.

Turner, J. D., Faulkner, H., Kamgno, J., Cormont, F., Snick, J. V., Else, K. J., Grencis, R. K., Behnke, J. M., Boussinesq, M. and Bradley, J. E. (2003). Th2 cytokines are associated with reduced worm burdens in a human intestinal helminth infection. Journal of Infectious Diseases 188(11): 1768-1775.

Tweyongyere, R., Mawa, P. A., Emojong, N. O., Mpairwe, H., Jones, F. M., Duong, T., Dunne, D. W., Vennervald, B. J., Katunguka-Rwakishaya, E. and Elliott, A. M. (2009). Effect of praziquantel treatment of *Schistosoma mansoni* during pregnancy on intensity of infection and antibody responses to schistosome antigens: results of a randomised, placebo-controlled trial. BMC Infectious Diseases 9: 32.

Ugbomoiko, U. S., Obiezue, R. N. N., Ogunniyi, T. A. B. and Ofoezie, I. E. (2009). Diagnostic accuracy of different urine dipsticks to detect urinary schistosomiasis: a comparative study in five endemic communities in Osun and Ogun States, Nigeria. Journal of Helminthology 83(3): 203-209.

Unutmaz, D. (2009). RORC2: The master of human Th17 cell programming. European Journal of Immunology 39(6): 1452-1455.

Urbani, C. and Albonico, M. (2003). AntI-helminthic drug safety and drug administration in the control of soil-transmitted helminthiasis in community campaigns. Acta Tropica 86(2-3): 215-221.

Usui, T., Nishikomori, R., Kitani, A. and Strober, W. (2003). GATA-3 Suppresses Th1 Development by Downregulation of Stat4 and Not through Effects on IL-12R[beta]2 Chain or T-bet. Immunity 18(3): 415-428.

van de Vijver, K. K., Deelder, A. M., Jacobs, W., van Marck, E. A. and Hokke, C. H. (2006). LacdiNAc- and LacNAc-containing glycans induce granulomas in an in vivo model for schistosome egg-induced hepatic granuloma formation. Glycobiology 16(3): 237-243.

van den Biggelaar, A. H., van Ree, R., Rodrigues, L. C., Lell, B., Deelder, A. M., Kremsner, P. G. and Yazdanbakhsh, M. (2000). Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. Lancet 356(9243): 1723-1727.

van den Biggelaar, A. H. J., Borrmann, S., Kremsner, P. and Yazdanbakhsh, M. (2002). Immune responses induced by repeated treatment do not result in protective immunity to *Schistosoma haematobium*: Interleukin (IL)–5 and IL-10 responses. Journal of Infectious Diseases 186(10): 1474-1482.

van der Does, A. M., Beekhuizen, H., Ravensbergen, B., Vos, T., Ottenhoff, T. H., van Dissel, J. T., Drijfhout, J. W., Hiemstra, P. S. and Nibbering, P. H. (2010). LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. Journal of Immunology 185(3): 1442-1449.

van der Kleij, D., Latz, E., Brouwers, J. F. H. M., Kruize, Y. C. M., Schmitz, M., Kurt-Jones, E. A., Espevik, T., de Jong, E. C., Kapsenberg, M. L., Golenbock, D. T., Tielens, A. G. M. and Yazdanbakhsh, M. (2002a). A novel host-parasite lipid cross-talk - Schistosomal lyso-phosphatidylserine activates Toll-like receptor 2 and affects immune polarization. Journal of Biological Chemistry 277(50): 48122-48129.

van der Kleij, D., van Remoortere, A., Schuitemaker, J. H. N., Kapsenberg, M. L., Deelder, A. M., Tielens, A. G. M., Hokke, C. H. and Yazdanbakhsh, M. (2002b). Triggering of innate immune responses by schistosome egg glycolipids and their carbohydrate epitope GalNAc beta 1-4(Fuc alpha 1-2Fuc alpha 1-3)GlcNAc. Journal of Infectious Diseases 185(4): 531-539.

van der Kleij, D., van den Biggelaar, A. H. J., Kruize, Y. C. M., Retra, K., Fillie, Y., Schmitz, M., Kremsner, P. G., Tielens, A. G. M. and Yazdanbakhsh, M. (2004). Responses to Toll-like receptor ligands in children living in areas where schistosome infections are endemic. Journal of Infectious Diseases 189(6): 1044-1051.

van der Werf, M. J., de Vlas, S. J., Brooker, S., Looman, C. W. N., Nagelkerke, N. J. D., Habbema, J. D. F. and Engels, D. (2003). Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. Acta Tropica 86(2-3): 125-139.

van Etten, L., Kremsner, P. G., Krijger, F. W. and Deelder, A. M. (1997). Day-to-day variation of egg output and schistosome circulating antigens in urine of *Schistosoma haematobium*-infected school children from Gabon and follow-up after chemotherapy. American Journal of Tropical Medicine and Hygiene 57(3): 337-341.

van Hellemond, J. J., Retra, K., Brouwers, J. F. H. M., van Balkom, B. W. M., Yazdanbakhsh, M., Shoemaker, C. B. and Tielens, A. G. M. (2006). Functions of the tegument of schistosomes: Clues from the proteome and lipidome. International Journal for Parasitology 36(6): 691-699.

van Leeuwen, B. H., Martinson, M. E., Webb, G. C. and Young, I. G. (1989). Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5. Blood 73(5): 1142-1148.

van Lieshout, L., Polman, K., Gryseels, B. and Deelder, A. M. (1998). Circulating anodic antigen levels in two areas endemic for schistosomiasis mansoni indicate differences in worm fecundity. Transactions of the Royal Society of Tropical Medicine and Hygiene 92(1): 115-119.

Varney, V. (1991). Hay-fever in the United-Kingdom. Clinical and Experimental Allergy 21(6): 757-762.

Varney, V. A., Gaga, M., Frew, A. J., Aber, V. R., Kay, A. B. and Durham, S. R. (1991). Usefulness of immunotherapy in patients with severe summer hay-fever uncontrolled by anti-allergic drugs. British Medical Journal 302(6771): 265-269.

Vasey, M. W. and Thayer, J. F. (1987). The continuing problem of false positives in repeated measures ANOVA in Psychophysiology: a multivariate solution. Psychophysiology 24(4): 479-486.

Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C. and Stockinger, B. (2008). Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. Nature Immunology 9(12): 1341-1346.

Verreck, F. A. W., de Boer, T., Langenberg, D. M. L., Hoeve, M. A., Kramer, M., Vaisberg, E., Kastelein, R., Kolk, A., de Waal-Malefyt, R. and Ottenhoff, T. H. M. (2004). Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2, macrophages subvert, immunity to (myco)bacteria. Proceedings of the National Academy of Sciences of the United States of America 101(13): 4560-4565.

Viana, I. R. C., Correaoliveira, R., Dossantos, O., Massara, C. L., Colosimo, E., Colley, D. G. and Gazzinelli, G. (1995). Comparison of antibody isotype responses to *Schistosoma mansoni* antigens by infected and putative resistant individuals living in an endemic area. Parasite Immunology 17(6): 297-304.

Vieira, L. Q., Colley, D. G., Desouza, C. P. S. and Gazzinelli, G. (1987). Stimulation of peripheral blood mononuclear cells from patients with schistosomiasis mansoni by living and fixed schistosomula and schistosomular membrane extracts and vesicles. American Journal of Tropical Medicine and Hygiene 36(1): 83-91.

Volpe, E., Servant, N., Zollinger, R., Bogiatzi, S. I., Hupe, P., Barillot, E. and Soumelis, V. (2008). A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human TH17 responses. Nature Immunology 9(6): 650-657.

von Ehrenstein, O. S., von Mutius, E., Illi, S., Baumann, L., Böhm, O. and von Kries, R. (2000). Reduced risk of hay fever and asthma among children of farmers. Clinical and Experimental Allergy 30(2): 187-193.

W.H.O. (2006). Preventive chemotherapy in human helminthiasis. Coordinated use of anthelminthic drugs in control interventions: a manual for health professionals and programme managers. G. W.H.O., W.H.O. Press**:** 1-56.

Wachholz, P. A., Nouri-Aria, K. T., Wilson, D. R., Walker, S. M., Verhoef, A., Till, S. J. and Durham, S. R. (2002). Grass pollen immunotherapy for hayfever is associated with increases in local nasal but not peripheral Th1:Th2 cytokine ratios. Immunology 105(1): 56-62.

Wagatsuma, Y., Aryeetey, Mary E., Sack, David A., Morrow, Richard H., Hatz, C. and Kojima, S. (1999). Resolution and resurgence of *Schistosoma haematobium* induced pathology after community-based chemotherapy in Ghana, as detected by ultrasound. Journal of Infectious Diseases 179(6): 1515-1522.

Walker, H. A. (2008). Floristics and physiognomy determine migrant landbird response to tamarisk (*Tamarix ramosissima*) invasion in riparian areas. The Auk 125(3): 520-531.

Walter, K., Fulford, A. J. C., McBeath, R., Joseph, S., Jones, F. M., Kariuki, H. C., Mwatha, J. K., Kimani, G., Kabatereine, N. B., Vennervald, B. J., Ouma, J. H. and Dunne, D. W. (2006). Increased human IgE induced by killing *Schistosoma mansoni in vivo* is associated with pretreatment Th2 cytokine responsiveness to worm antigens. Journal of Immunology 177(8): 5490-5498.

Wamachi, A. N., Mayadev, J. S., Mungai, P. L., Magak, P. L., Ouma, J. H., Magambo, J. K., Muchiri, E. M., Koech, D. K., King, C. H. and King, C. L. (2004). Increased ratio of tumor necrosis factor-alpha to interleukin-10 production is associated with *Schistosoma haematobium*-induced urinary-tract morbidity. Journal of Infectious Diseases 190(11): 2020-2030.

Watanabe, K., Mwinzi, P. N., Black, C. L., Muok, E. M., Karanja, D. M., Secor, W. E. and Colley, D. G. (2007). T regulatory cell levels decrease in people infected with *Schistosoma mansoni* on effective treatment. American Journal of Tropical Medicine and Hygiene 77(4): 676-682.

Webster, M., Correa-Oliveira, R., Gazzinelli, G., Viana, I. R. C., Fraga, L. A. D. O., Silveira, A. M. S. and Dunne, D. W. (1997a). Factors affecting high and low human IgE responses to schistosome

worm antigens in an area of Brazil endemic for *Schistosoma mansoni* and hookworm. American Journal of Tropical Medicine and Hygiene 57(4): 487-494.

Webster, M., Libranda-Ramirez, B. D., Aligui, G. D., Olveda, R. M., Ouma, J. H., Kariuki, H. C., Kimani, G., Olds, G. R., Fulford, A. J., Butterworth, A. E. and Dunne, D. W. (1997b). The influence of sex and age on antibody isotype responses to Schistosoma mansoni and Schistosoma japonicum in human populations in Kenya and the Philippines. Parasitology 114 (Pt 4): 383-393.

Wilcoxon, F. (1945). Individual comparisons by ranking methods. Biometrics Bulletin 1(6): 80-83.

Wilson, M. S. and Maizels, R. M. (2004). Regulation of allergy and autoimmunity in helminth infection. Clinical Reviews in Allergy and Immunology 26(1): 35-50.

Wilson, M. S., Taylor, M. D., Balic, A., Finney, C. A. M., Lamb, J. R. and Maizels, R. M. (2005). Suppression of allergic airway inflammation by helminth-induced regulatory T cells. Journal of Experimental Medicine 202(9): 1199-1212.

Wilson, N. J., Boniface, K., Chan, J. R., McKenzie, B. S., Blumenschein, W. M., Mattson, J. D., Basham, B., Smith, K., Chen, T., Morel, F., Lecron, J.-C., Kastelein, R. A., Cua, D. J., McClanahan, T. K., Bowman, E. P. and de Waal Malefyt, R. (2007). Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nature Immunology 8(9): 950-957.

Wilson, R. A. and Coulson, P. S. (2006). Schistosome vaccines: a critical appraisal. Memorias do Instituto Oswaldo Cruz 101 Suppl 1: 13-20.

Wilson, S., Jones, F. M., Mwatha, J. K., Kimani, G., Booth, M., Kariuki, H. C., Vennervald, B. J., Ouma, J. H., Muchiri, E. and Dunne, D. W. (2008). Hepatosplenomegaly is associated with low regulatory and Th2 responses to schistosome antigens in childhood schistosomiasis and malaria coinfection. Infection and Immunity 76(5): 2212-2218.

Woolhouse, M. E. J., Watts, C. H. and Chandiwana, S. K. (1991). Heterogeneities in transmission rates and the epidemiology of schistosome infection. Proceedings of the Royal Society of London Series B-Biological Sciences 245(1313): 109-114.

Woolhouse, M. E. J. (1992). A theoretical framework for the immunoepidemiology of helminth infection. Parasite Immunology 14(6): 563-578.

Woolhouse, M. E. J. (1994). Immunoepidemiology of human schistosomes: Taking the theory into the field. Parasitology Today 10(5): 196-202.

Woolhouse, M. E. J. (1998). Patterns in parasite epidemiology: The peak shift. Parasitology Today 14(10): 428-434.

Woolhouse, M. E. J. and Hagan, P. (1999). Seeking the ghost of worms past. Nature Medicine 5(11): 1225-1227.

Wosinska-Becler, K., Plewako, H., Håkansson, L. and Rak, S. (2004). Cytokine production in peripheral blood cells during and outside the pollen season in birch-allergic patients and non-allergic controls. Clinical and Experimental Allergy 34(1): 123-130.

Wright, V. and Bickle, Q. (2005). Immune responses following experimental human hookworm infection. Clinical and Experimental Immunology 142(2): 398-403.

Wurster, A. L., Rodgers, V. L., Satoskar, A. R., Whitters, M. J., Young, D. A., Collins, M. and Grusby, M. J. (2002). Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon γ–producing Th1 Cells. Journal of Experimental Medicine 196(7): 969-977.

Wynn, T. A., Eltoum, I., Oswald, I. P., Cheever, A. W. and Sher, A. (1994). Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. Journal of Experimental Medicine 179(5): 1551-1561.

Wynn, T. A. and Cheever, A. W. (1995). Cytokine regulation of granuloma formation in schistosomiasis. Current Opinion in Immunology 7(4): 505-511.

Wynn, T. A., Cheever, A. W., Jankovic, D., Poindexter, R. W., Caspar, P., Lewis, F. A. and Sher, A. (1995a). An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. Nature 376(6541): 594-596.

Wynn, T. A., Jankovic, D., Hieny, S., Zioncheck, K., Jardieu, P., Cheever, A. W. and Sher, A. (1995b). IL-12 exacerbates rather than suppresses T helper 2-dependent pathology in the absence of endogenous IFN-gamma. Journal of Immunology 154(8): 3999-4009.

Xu, C. B., Verwaerde, C., Grzych, J. M., Fontaine, J. and Capron, A. (1991). A monoclonal antibody blocking the *Schistosoma mansoni* 28-kDa glutathione S-transferase activity reduces female worm fecundity and egg viability. European Journal of Immunology 21(8): 1801-1807.

Xu, C. B., Verwaerde, C., Gras-Masse, H., Fontaine, J., Bossus, M., Trottein, F., Wolowczuk, I., Tartar, A. and Capron, A. (1993). *Schistosoma mansoni* 28-kDa glutathione S-transferase and immunity against parasite fecundity and egg viability. Role of the amino- and carboxyl-terminal domains. Journal of Immunology 150(3): 940-949.

Xu, X., Stack, R. J., Rao, N. and Caulfield, J. P. (1994). *Schistosoma mansoni*: fractionation and characterization of the glycocalyx and glycogen-like material from cercariae. Experimental Parasitology 79(3): 399-409.

Xu, Y.-Z., Matsuda, H. and Dresden, M. H. (1988). Effect of praziquantel on *Schistosoma mansoni* eggs: Leucine Aminopeptidase (LAP) activity and anti-LAP antibodies. American Journal of Hygiene and Tropical Medicine 39(1): 46-51.

Yazdanbakhsh, M., Kremsner, P. G. and van Ree, R. (2002). Allergy, parasites, and the hygiene hypothesis. Science 296(5567): 490-494.

Yazdanbakhsh, M. and Sacks, D. L. (2010). Why does immunity to parasites take so long to develop? Nature Reviews Immunology 10(2): 80-81.

Yemaneberhan, H., Bekele, Z., Venn, A., Lewis, S., Parry, E. and Britton, J. (1997). Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. Lancet 350(9071): 85-90.

Zhang, W. B., Ross, A. G. and McManus, D. P. (2008). Mechanisms of immunity in hydatid disease: Implications for vaccine development. Journal of Immunology 181(10): 6679-6685.

Zhou, Y., Zheng, H. J., Chen, Y. Y., Zhang, L., Wang, K., Guo, J., Huang, Z., Zhang, B., Huang, W., Jin, K., Dou, T. H., Hasegawa, M., Wang, L., Zhang, Y., Zhou, J., Tao, L., Cao, Z. W., Li, Y. X., Vinar, T., Brejova, B., Brown, D., Li, M., Miller, D. J., Blair, D., Zhong, Y., Chen, Z., Hu, W., Wang, Z. Q., Zhang, Q. H., Song, H. D., Chen, S. J., Xu, X. N., Xu, B., Ju, C., Huang, Y. C., Brindley, P. J., McManus, D. P., Feng, Z., Han, Z. G., Lu, G., Ren, S. X., Wang, Y. Z., Gu, W. Y., Kang, H., Chen, J., Chen, X. Y., Chen, S. T., Wang, L. J., Yan, J., Wang, B. Y., Lv, X. Y., Jin, L., Wang, B. F., Pu, S. Y., Zhang, X. L., Zhang, W., Hu, Q. P., Zhu, G. F., Wang, J., Yu, J., Wang, J., Yang, H. M., Ning, Z. M., Beriman, M., Wei, C. L., Ruan, Y. J., Zhao, G. P., Wang, S. Y., Liu, F., Wang, Z. Q., Zheng, H. J., Zhang, Q. H., Wang, S. Y., Han, Z. G. and Seque, *S. j.* G. (2009). The *Schistosoma japonicum* genome reveals features of host-parasite interplay. Nature 460(7253): 345-U356.

Appendices

Appendix 1

Magaya community baseline population census questionnaire. Questionnaire administered to all participants at recruitment in September 2008 to assess sex, age, date of birth, residential history, treatment history, water contact behaviour and *a priori* knowledge of schistosomiasis and malaria. Questionnaires were administered on a one-to-one basis in the local language (Shona) by a native speaker.

Appendix 2

Magnitude of *S. haematobium* egg, adult worm, GST and MBP-specific cytokine responses and cytokine concentrations present in un-stimulated cultures of the cohort analysed in chapter 4 (n = 198). Mean of antigens-specific cytokine responses (un-transformed) after subtraction of concentrations present in un-stimulated cultures are given alongside un-transformed means of raw cytokine concentations present in un-stimulated cultures. S.E.M.: Standard error of the mean

Appendix 3a

Immunological characteristics of the plasma cohort differ between *T. suis* and placebo-treated participants after, but not before initial treatment (n = 89). Results of ANOVA comparisons between treatment groups at each timepoint. Exploratory analysis verified that un-transformed data and residuals of ANOVA models met parametric assumptions. Significant differences ($p<0.05$) are highlighted in bold and those significant after Bonferroni adjustment are shaded grey. df – degrees of freedom.

Appendix 3b

Immunological characteristics of the PBMC supernatant cohort differ between *T. suis* and placebo-treated participants after, but not before initial treatment (n = 22). Results of ANOVA comparisons between treatment groups at each timepoint. Exploratory analysis verified that un-transformed data and residuals of ANOVA models met parametric assumptions. Significant differences ($p<0.05$) are highlighted in bold and those significant after Bonferroni adjustment are shaded grey. df – degrees of freedom.

Appendix 4

Diagnostic statistics of final NMS solutions for pre and 6 weeks post-treatment cytokine responses to *S. haematobium* antigens. All values were consistent with the *a priori* stress criteria for stable ordination solutions (final stress value <20) obtained at or within 500 iterations. All solutions were also achieved with a final stress value that was significantly lower than that obtained from randomly assorted data as assessed by Monte-Carlo randomisation tests ($p<0.05$).

Appendix 5

Publications of work related to this thesis.

Appendix 1.

Bilharzia Questionnaire

1. **This questionnaire is 4 pages long- please complete ALL pages.**
2. ***Questionnaire to be completed at the time of enrolment of children into the study and only after obtaining parents' consent and assent of participating child.***

Date of questionnaire_____
Person administering questionnaire_____
Parent's name _____

Participant Name_____ **ID #**_____
Gender M F ***(circle one)***
Date of birth (DD MM YY)_____ **Age**_____(years)
Age group: 7-10 11-14 15-17 18-25 26-35 36+ ***(circle one)***
Body weight _____kg **Height** _____cm
School _____ **Village**
Name_____

Have you lived in this village all your life? Y N **(*circle either yes or no*)**
If no, **which other villages have you lived in and for how long?**
(complete in order of residence)

	Village name	**District**	**How long (years)?**
1 (Born here)	_____	_____	_____
2.	_____	_____	_____
3.	_____	_____	_____
3.	_____	_____	_____

Which primary school did you attend? _____
Which secondary school did you attend? _____
Do you make regular visits to other villages? Y N ***(circle either yes or no*)**

If yes, Where? _____
When? _____
Do you participate in sports? Y N ***(circle either yes or no*)**
If no, why not? _____
Have you ever had Schistosomiasis (bilharzia) infection? Yes / No / Do not know ***(circle one)***

If yes, were you treated? Yes / No / Do not know ***(circle one)***

If yes, do you remember when you were treated?_____ How?_____
Have you ever had Malaria? Yes / No / Do not know ***(circle one)***
If yes, were you treated? Yes / No / Do not know ***(circle one)***
If yes, do you remember when you were treated?_____ How?_____

Other Signs and symptoms (ask then prompt from list)
Haematuria (Blood in urine) _____

Dysuria (Difficulty in urination)_____

Frequent urge to urinate (approximate estimate)_____

Fever I (37.5-38.5^{0}C) II (38.6-40^{0}C) III (40^{0}C+)

Chills_____

Arthralgia (Pain in joints) _____ Myalgia (Muscle pain) _____

Backache _____ Lethargy (Sleepy)_____

Diarrhoea:_____

Other symptoms_____

Splenomegaly (enlarged spleen) _____

Hepatomegaly (enlarged liver) _____

Hepatitis B antibody (if known)_____

Which village were you born in?_____

(check this matches village they live in now **–** if not check have full list of all villages lived in)

The following information is being collected to assess various likely or probable risk factors associated with malaria and / or schistosomiasis infection

A. Domestic water source ***(circle as many as appropriate)***
1 unprotected well 2 river 3 dam 4 Upgraded well 5 Borehole
6 Tap 7 Other (specify) _____

B. Where do you normally go to the toilet? ***(circle as many as appropriate)***
1 bush 2 cat sanitation 3 Latrine/toilet 4.Other (specify) _____

C. Is there a latrine at your home? Y N ***(circle either yes or no***)

D. If yes, are there any problems in using it? Y N ***(circle either yes or no***)
If yes, explain _____

E. Do you pass through water on your way to and from school? Y N ***(circle one)***
If yes, where? (describe and/or give site name)_____

F. Water contact activities

	Yes/No (code)		How many times?	Where?*				At what time?* (e.g. 0800, 1200, 1600) (code)	
				River/Dam/Well/Other (if Other, specify) (code)		If River or Dam, name of place or nearest village/school (code)			
(1) Did you go swimming yesterday?									
(2) Did you play in the water yesterday?									
(3) Did you go to bathe yesterday?									
(4) Did you do laundry yesterday?									
(5) Did you wash dishes yesterday?									
(6) Did you go to wash yesterday? (face and legs)									
(7) Did you collect water yesterday?									
(8) Did you go fishing yesterday?									
(9) Did you cross any rivers yesterday?									
(10) Did you go to the water for any other reason yesterday?									
(10 a) If answer to Q10 **yes**: What did you do?_____									

***Answer for <u>each contact</u>**

G. In the last 7 days, have you carried out any of these activities at the river or dam? ***(circle as many as appropriate*)**

	If yes, how many		**If yes, how many**
Swimming	_____	Washing (face and legs)	_____
Playing in the	_____	Collecting water	_____
Bathing	_____	Fishing	_____
Laundry	_____	Crossing river	_____
Washing dishes	_____	Other_____	_____

The following questions are simply to assess knowledge of the participants and are not to be used for inclusion / exclusion)

H. Do you know what bilharzia is? Y N ***(circle either yes or no*)**

If yes, where did you learn about bilharzia? *(circle as many as appropriate*)

1. School
2. Health centre
3. Community
4. Family
5. Read book
6. Nowhere
7. Other _____

I. Do you know what the symptoms of bilharzia are? *(circle as many as appropriate*)

1. Blood in urine
2. Tiredness
3. Mental illness
4. Pain upon urination
5. Other _____

J. Do you know how bilharzia is spread? *(circle as many as appropriate*)

1. Urinating in water
2. Defecating in water
3. Swimming
4. Stepping on urine
5. Drinking dirty water
6. Snails
7. Other _____

K. Do you know what the long-term effects of bilharzia are? *(circle as many as appropriate*)

1. Infertility
2. Stomach/bladder damage
3. Death
4. Going mad
5. Don't know

6. Other _____

L. Do you try to protect yourself from getting bilharzia? Y N ***(circle one***)

If yes, how? _____

If no, why not? ***(circle as many as appropriate*****)**

Economic reasons

Distance to clinic

Lack of knowledge of treatment options,

Religious reasons

Other _____

M. Do you have any questions or comments concerning this study?

Appendix 2.

		WWH*	SEA*	GST*	MBP*	Media
	n	**198**	**198**	**198**	**198**	**198**
TNFα	**Mean**	0.017	0.015	0.019	0.265	0.016
	S.E.M.	0.006	0.004	0.007	0.027	0.004
IL-6	**Mean**	0.382	0.599	0.621	6.650	0.215
	S.E.M.	0.101	0.103	0.097	0.426	0.043
IL-8	**Mean**	0.094	0.387	0.152	0.719	0.862
	S.E.M.	0.017	0.068	0.025	0.103	0.134
IFNγ	**Mean**	0.043	0.046	0.081	0.460	0.050
	S.E.M.	0.017	0.013	0.033	0.090	0.021
IL-2	**Mean**	0.009	0.008	0.013	0.006	0.020
	S.E.M.	0.003	0.002	0.004	0.003	0.005
IL-12p70	**Mean**	0.015	0.014	0.023	0.115	0.010
	S.E.M.	0.006	0.004	0.007	0.022	0.002
IL-4	**Mean**	0.001	0.001	0.000	0.000	0.002
	S.E.M.	0.000	0.000	0.000	0.000	0.000
IL-5	**Mean**	0.010	0.014	0.010	0.007	0.030
	S.E.M.	0.002	0.004	0.002	0.002	0.005
IL-10	**Mean**	0.009	0.012	0.014	0.029	0.026
	S.E.M.	0.003	0.003	0.004	0.005	0.007
IL-13	**Mean**	0.031	0.026	0.025	0.022	0.126
	S.E.M.	0.011	0.006	0.007	0.010	0.037
IL-17A	**Mean**	0.001	0.002	0.002	0.002	0.006
	S.E.M.	0.000	0.001	0.001	0.001	0.002
IL-21	**Mean**	0.071	0.081	0.069	0.083	0.428
	S.E.M.	0.013	0.019	0.009	0.018	0.111
IL-23	**Mean**	0.040	0.048	0.066	0.654	0.028
	S.E.M.	0.012	0.010	0.014	0.051	0.006

*cytokine concentrations present in un-stimulated cultures (Media) subtracted

Appendix 3a.

Immune Response	*Baseline (df:1, error df: 88)*			*Grass Pollen Season (df:1, error df: 87)**			*End (df:1, error df: 87)**		
	F	*p*	*Post-hoc comparisons*	*F*	*p*	*Post-hoc comparisons*	*F*	*p*	*Post-hoc comparisons*
Eosinophil count	0.035	0.853		20.366	**<0.001**	***T. suis*>Placebo**	21.256	**<0.001**	***T. suis*>Placebo**
Lymphocyte count	0.096	0.757		0.823	0.367		0.413	0.522	
Total IgE	0.153	0.697		0.126	0.723		0.067	0.797	
Serum Histamine	0.051	0.821		0.214	0.644		0.684	0.410	
g6 IgE	0.260	0.612		0.059	0.809		0.008	0.928	
g6 IgG	0.196	0.659		6.599	**0.012**	***T. suis*>Placebo**	5.696	**0.019**	***T. suis*>Placebo**
g6 IgG4	0.012	0.912		2.208	0.141		1.236	0.269	
E\S IgA	0.556	0.458		17.547	**<0.001**	***T. suis*>Placebo**	12.508	**0.001**	***T. suis*>Placebo**
E\S IgE	0.798	0.374		9.115	**0.003**	***T. suis*>Placebo**	13.404	**<0.001**	***T. suis*>Placebo**
E\S IgG	0.492	0.485		55.936	**<0.001**	***T. suis*>Placebo**	94.127	**<0.001**	***T. suis*>Placebo**
E\S IgG4	0.181	0.671		8.637	**0.004**	***T. suis*>Placebo**	30.967	**<0.001**	***T. suis*>Placebo**

**** 1 case missing data***

Appendix 3b.

Immune Response	*Baseline (df: 1, error df: 20)*			*Grass Pollen Season (df: 1, error df: 19)**			*End (df:1, error df: 19)**		
	F	*p*	*Post-hoc comparisons*	*F*	*p*	*Post-hoc comparisons*	*F*	*p*	*Post-hoc comparisons*
Eosinophil count	0.176	0.679		5.181	**0.035**	***T. suis*>Placebo**	5.602	**0.029**	***T. suis*>Placebo**
Lymphocyte count	0.100	0.755		0.554	0.466		1.039	0.321	
Total IgE	0.039	0.845		0.101	0.755		1.564	0.226	
Serum Histamine	0.953	0.341		0.226	0.640		6.818	**0.017**	***T. suis*>Placebo**
g6 IgE	0.294	0.593		0.039	0.845		0.350	0.561	
g6 IgG	0.047	0.830		1.703	0.207		0.529	0.476	
g6 IgG4	0.003	0.954		0.001	0.970		0.015	0.905	
E\S IgA	1.471	0.239		9.823	**0.005**	***T. suis*>Placebo**	5.334	**0.032**	***T. suis*>Placebo**
E\S IgE	0.051	0.824		6.296	**0.021**	***T. suis*>Placebo**	5.300	**0.033**	***T. suis*>Placebo**
E\S IgG	0.018	0.894		16.518	**0.001**	***T. suis*>Placebo**	12.802	**0.002**	***T. suis*>Placebo**
E\S IgG4	0.000	0.691		10.382	**0.004**	***T. suis*>Placebo**	5.920	**0.025**	***T. suis*>Placebo**

**** 1 case missing data***

Appendix 4

Antigen	*Dimensions*	*Final stress*	*Final instability*	*Number of iterations*
CAP	2	9.5	<0.000001	92
WWH	1	18.08	0.00001	500
SEA	2	7.03	<0.000001	118
GST	2	9.59	0.00021	500
Un-stimulated	2	8.5	<0.000001	290

Appendix 5

Bourke, C.D., Maizels, R.M. and Mutapi, F. (2010). Acquired immune heterogeneity and its sources in human helminth infection. Parasitology: 138 (2): 139-159*

Imai, N., Rujeni, N., Nausch, N., Bourke, C.D., Appleby, L.J., Cowan, G., Gwisai, R., Midzi, N., Cavanagh, D., Mduluza, T., Taylor, D. and Mutapi, F. (2011). Exposure, infection, systemic cytokine levels and antibody responses in young children concurrently exposed to schistosomiasis and malaria. Parasitology: 138 (12): 1519-1533

Mutapi, F., Bourke, C.D., Harcus, Y., Midzi, N., Mduluza, T., Turner, C. M. and Maizels, R.M. (2011a). Differential recognition of *Schistosoma haematobium* adult worm antigens by the human antibodies IgA, IgE, IgG1 and IgG4. Parasite Immunology: 33 (3): 181-192.

Mutapi, F., Imai, N., Nausch, N., Bourke, C.D., Rujeni, N., Mitchell, K.M., Midzi, N., Woolhouse, M.E.J., Maizels, R.M. and Mduluza, T. (2011b). Schistosome infection intensity is inversely related to auto-reactive antibody levels. PLoS ONE: 6 (5): e19149

Mutapi, F., Rujeni, N., Bourke, C.D., Mitchell, K.M., Appleby, L., Nausch, N., Midzi, N., Woolhouse, M.E.J., Maizels, R.M. and Mduluza, T. (2011c). *Schistosoma haematobium* treatment in 1-5 year old children: Safety and efficacy of the anti-helminthic drug praziquantel. PLoS Neglected Tropical Diseases: 5 (5): e1143

*a copy is provided overleaf

REVIEW ARTICLE

Acquired immune heterogeneity and its sources in human helminth infection

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SUMMARY

Similarities in the immunobiology of different parasitic worm infections indicate that co-evolution of humans and helminths has shaped a common anti-helminth immune response. However, recent *in vitro* and immuno-epidemiological studies highlight fundamental differences and plasticity within host-helminth interactions. The 'trade-off' between immunity and immunopathology inherent in host immune responses occurs on a background of genetic polymorphism, variable exposure patterns and infection history. For the parasite, variation in life-cycle and antigen expression can influence the effector responses directed against them. This is particularly apparent when comparing gastrointestinal and tissue-dwelling helminths. Furthermore, insights into the impact of anti-helminthic treatment and co-infection on acquired immunity suggest that immune heterogeneity arises not from hosts and parasites in isolation, but also from the environment in which immune responses develop. Large-scale differences observed in the epidemiology of human helminthiases are a product of complex host-parasite-environment interactions which, given potential for exposure to parasite antigens *in utero*, can arise even before a parasite interacts with its human host. This review summarizes key differences identified in human acquired immune responses to nematode and trematode infections of public health importance and explores the factors contributing to these variations.

Key words: heterogeneity, helminth, human, nematode, trematode, schistosome, immune response.

INTRODUCTION

Over a third of the human population is currently infected by one or more species of parasitic helminth (Hotez *et al*. 2008). Chronically-infected hosts must strike a balance between anti-parasite protective responses and limiting immune-mediated pathology (Hoffmann *et al*. 2002), whilst parasites have developed strategies to prolong intra-host survival and fecundity. Throughout their co-evolutionary history these forces, as often in concert as in opposition, have driven diversity in both parasites (Maizels *et al*. 2001) and the genetics of the host immune response (Fumagalli *et al*. 2009; Maizels, 2009), the latter especially evident in the human population. Experimental models are invaluable in mechanistic studies of helminth-induced immune responses *in vivo*; however, reductionist laboratory models inevitably seek to minimize variation in the host, the context of infection and polymorphism in the parasite itself. Moreover, integral differences in the immune systems of different host species (Mestas and Hughes, 2004) and the inability of many anthropophilic helminths to naturally infect laboratory animals mean that current experimental models do not reflect the complexity of human helminth immunobiology. Most importantly, models cannot easily replicate the major sources of human immune heterogeneity such as parasite transmission dynamics (Mutapi *et al*. 1997), distribution of intermediate hosts (Gryseels *et al*. 2006), distinct intra-population exposure patterns (Rudge *et al*. 2008), therapeutic interventions (Mutapi *et al*. 2005) and concurrent or previous infections with other pathogen species (Correa-Oliveira *et al*. 2002).

This review highlights differences in the human acquired immune response to a variety of nematode and trematode infections of public health importance and explores some of the factors contributing to these variations, particularly differences arising from helminth life-history traits.

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COMMON FEATURES OF THE ACQUIRED IMMUNE RESPONSE

The most prevalent human helminthiases are caused by nematode species including filarial worms (*Brugia*

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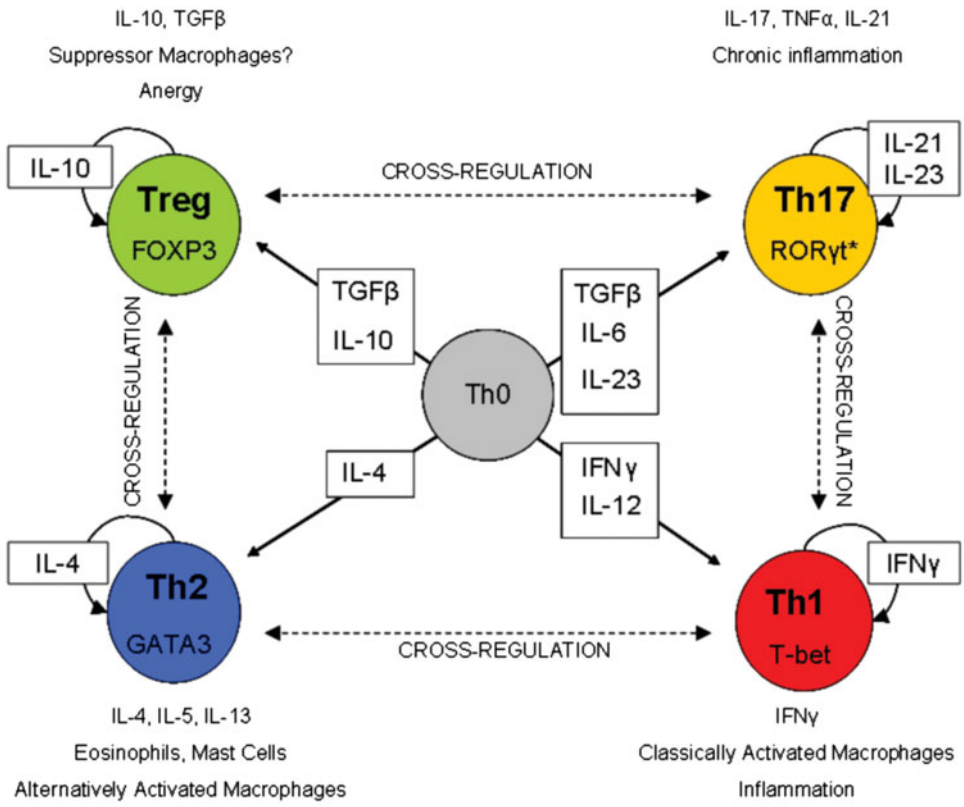

Fig. 1. Summary of the major CD4+ T cell differentiation pathways following activation in the periphery. Cytokines and transcription factors involved in T cell polarization are shown in boxes and within cells respectively. Effector cell types and cytokines associated with each CD4+ T cell phenotype are given adjacent to relevant cells. Th0 – naïve T cell, * RORγt – murine transcription factor, the human orthologue is RORC2. Figure adapted from Deenick and Tangyne (2007) and Diaz and Allen (2007).

malayi, *Onchocerca volvulus* and *Wuchereria bancrofti*) and soil-transmitted helminths (Hotez *et al*. 2008): *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm (*Ancyclostoma duodenale* and *Necator americanus*), *Strongyloides* spp., and *Enterobius vermicularis*. Of the trematodes, *Schistosoma* spp. are of greatest public health importance, with the 3 predominant species (*S. haematobium*, *S. japonicum* and *S. mansoni*) accounting for over 200 million current infections worldwide (Gryseels *et al*. 2006). In addition, and beyond the scope of this article, there are many important human cestode parasites which have been expertly discussed elsewhere (Zhang *et al*. 2008).

The principal cellular mediators of human-helminth interactions are $CD4^+$ T cells, which can differentiate into alternative lineages once activated; Th1, Th2, Th17 and T regulatory (Treg), as summarized in Fig. 1. Selective differentiation is first driven by innate antigen-presenting cells (APC) but, as responses mature during the course of chronic infection, cytokine-mediated cross-regulation between T cell subsets becomes increasingly important. Differences in acquired immune responses to helminth infection can arise via heterogeneity in how parasites initially interact with host cells, polarize local and systemic responses and/or modulate effector responses in chronic infection. There are two principal immunological features believed to be common amongst helminth infections. (1) Polarization of $CD4^+$ T cells towards a Th2 phenotype. In humans, this phenotype is associated with production of interleukins (IL-)4, 5, 9, 10 and 13 (Turner *et al*. 2003; Jackson *et al*. 2004*b*; Quinnell *et al*. 2004), secretion of IgE and IgG4 isotypes by plasma cells (Hagan *et al*. 1991) and activation of downstream effector cells such as eosinophils (reviewed by (Klion and Nutman, 2004)). (2) Immunosuppression of both worm-specific and generalized immune responses. Inducible mechanisms include secretion of suppressive cytokines, such as IL-10 and TGF-β, and expansion of regulatory cell populations, particularly Tregs (Doetze *et al*. 2000; Watanabe *et al*. 2007; Babu *et al*. 2009). The progression of host responses from effector Th2 to a so-called 'modified Th2' phenotype associated with elevated levels of Treg-associated molecules and reduced Th2 effector cytokine responses (Maizels and Yazdanbakhsh, 2003), suggests that both features play a functional, and potentially cross-regulatory, role in helminth immunobiology.

Despite similarities in the immune responses elicited by different helminths, immunological studies often yield contradictory results in different human populations and different species of helminth infection, which are discussed below. To an extent this is

unsurprising as expanding research into human helminthiases has inevitably led to a greater appreciation of the intricacies of their immunobiology. The discovery of new cell populations including Treg and Th17 and the more recent description of IL-5 and IL-9 producing CD4+ T cells, which differentiate from Th2 cells in an IL-33 or TGF-β/IL-4-dependent manner respectively, has led to a re-evaluation of the classical Th1-Th2 paradigm (Dardalhon *et al*. 2008; Kurowska-Stolarska *et al*. 2008; Veldhoen *et al*. 2008). Furthermore the CD4+ T cell axis can be regulated both by effector cytokines from CD4+ T cell populations themselves (IFNγ, IL-4 and IL-21 (Wilson *et al*. 2008; Babu *et al*. 2009)) and innate and adaptive non-T cell populations, which both present antigen to activate T cells and contribute significant levels of these same cytokines. Helminth-induced alternatively activated macrophages exhibit particular plasticity in this respect (Jenkins and Allen, 2010). Studies showing that Th2 IL-4 and IL-5 responses can be dissociated in human nematode (Sartono *et al*. 1997) and trematode (Scott *et al*. 2000) infections indicate that even established immune phenotypes involve heterogeneous effector responses to helminth infection. As more data become available on human anti-helminth responses, particularly in terms of the more recently described cytokines (e.g. IL-17, IL-21, IL-23, IL-33), comparative studies between helminth groups will be better able to identify similarities and differences in these responses.

HETEROGENEITY IN PROTECTIVE IMMUNE RESPONSES

Protective immunity in human helminthiases encompasses a range of overlapping mechanisms: (a) complete elimination of parasites (sterile immunity), (b) resistance to *de novo* infection (non-sterile immunity, also called 'concomitant immunity') and (c) reduction of immunopathology (tolerance). The former two processes can be grouped as anti-parasite immunity, whilst the latter involves immune-mediated regulation of pathological effector responses. The high prevalence of chronic helminth infection in endemic populations suggests that sterile immunity is rarely generated (Hotez *et al*. 2008). However, the decline in infection intensity at an earlier age in populations with high infection intensity (Woolhouse, 1998) and more rapid development of resistance to re-infection post-treatment in individuals with long-term exposure to infection (Black *et al*. 2010) indicates that non-sterile immunity, though slow to develop, does occur with cumulative exposure. Conversely, since the majority of helminth infections are asymptomatic, it is clear that tolerance of low-level infection can be readily elicited to limit immunopathology (Dessein *et al*. 2004). The balance between anti-parasite and anti-pathology responses is inevitably shaped by the specific immunopathogenesis of different helminth species. Contrary to phylogenetic distinctions between nematodes and trematodes, one of the main functional delineations between helminth infections is that between species where adult worms reside in the gastrointestinal (GI) tract and those that inhabit host tissues.

Anti-parasite immune responses

Expulsion of GI nematodes is dependent on highly polarised Th2 responses (reviewed by (Jackson *et al*. 2009; Jenkins and Allen, 2010)) and elevated titres of systemic and parasite-specific Th2 cytokines are negatively associated with infection intensity in untreated populations (Turner *et al*. 2003). Th2-induced smooth muscle hypercontractility and mucus secretion by goblet cells are known to facilitate clearance of murine GI nematode infections (Finkelman *et al*. 2004), but these physical means of worm expulsion are absent in the tissues. The immunological relevance of these differences has not been tested in humans.

Resistance to re-infection by GI nematodes post-treatment also tends to be unequivocally Th2-mediated (Jackson *et al*. 2004*a*,*b*; Quinnell *et al*. 2004). Post-treatment resistance to hookworm and *T. trichiura* infection is associated with pre-treatment levels of IL-5 (Jackson *et al*. 2004*a*; Quinnell *et al*. 2004) and negative associations have also been shown between IL-5/IL-13 and re-infection with *A. lumbricoides* and *T. trichiura* (Jackson *et al*. 2004*b*). However, even between GI nematode species the relationship between Th2 cytokines and resistance to re-infection has been shown to vary according to parasite life-history. For example despite multiple shared risk factors for co-infection with *A. lumbricoides* and *T. trichiura*, pre-treatment Th2 cytokine titres were only associated with post-treatment resistance to the latter (Jackson *et al*. 2004*a*). Furthermore, parasite-specific IL-10 was found to be an indicator of species-specific susceptibility, being negatively associated with *T. trichiura* but positively associated with *A. lumbricoides* egg counts (Jackson *et al*. 2004*a*).

Th2-type responses are also involved in protective immunity in tissue-dwelling helminths; however, chronically infected individuals tend to mount Th1-Th2 mixed responses (Joseph *et al*. 2004; Mutapi *et al*. 2007), thought to be involved in limiting infection intensity (Turaga *et al*. 2000). In an *O. volvulus*-endemic population in Cameroon, despite a predominant Th2 bias, putative immunity correlated with elevated titres of parasite-specific Th1 (IFNγ), Th2 (IL-5) and innate (granulocyte-macrophage colony-stimulating factor (GM-CSF)) effector cytokines (Turaga *et al*. 2000). Similarly, schistosome-specific IFNγ production by peripheral

blood mononuclear cells (PBMC) correlates with immunity to schistosomiasis (Viana *et al*. 1994). Mixed Th1-Th2 cytokine responses are observed in chronic trichuriasis in the gut but have not been significantly associated with infection intensity (Faulkner *et al*. 2002).

Antibody-mediated protection from helminths is typically attributed to elevated titres of parasite-specific IgE (Faulkner *et al*. 2002; Pearce and MacDonald, 2002), whilst a low IgE:IgG4 is associated with susceptibility to schistosome (Hagan *et al*. 1991) and GI infections (Figueiredo *et al*. 2010). However, positive correlations between parasite-specific IgE and other isotypes (Viana *et al*. 1995) and studies identifying more pronounced associations between infection intensity and non-IgE isotypes (Webster *et al*. 1997) suggest that there is redundancy in antibody-mediated protective immunity. In addition, given the potentially immunopathogenic outcomes of IgE-mediated cellular effector responses (Nutman and Kumaraswami, 2001; Cooper *et al*. 2004), it is unsurprising that propagation of alternate antibody isotypes in chronically infected hosts may be favourable. A variety of studies investigating other antibody isotypes in schistosomiasis provide conflicting evidence for their role in resistance to infection with different species. For example, whilst polyclonal adult worm-specific IgA titres decline with age and infection intensity in *S. haematobium* endemic areas (Mutapi *et al*. 1997), the opposite pattern has been observed for *S. japonicum*-specific IgA in the Philippines (Acosta *et al*. 2004). Similarly, whilst the former study found that *S. haematobium* adult worm-specific IgG1 increased with age and peaked in individuals with low infection intensity (Mutapi *et al*. 1997), IgG1 titres were significantly positively associated with intensity of *S. mansoni* infection in Brazil (de Jesus *et al*. 2000) and Kenya (Naus *et al*. 1999). The role of IgM in anti-schistosome responses is also controversial as adult worm and egg-specific IgM increase with *S. haematobium* and *S. japonicum* infection intensity in some populations (Mutapi *et al*. 1997; Acosta *et al*. 2004), but adult-worm-specific titres were lowest in individuals patently infected with *S. mansoni* elsewhere (Viana *et al*. 1995; Caldas *et al*. 2000). Potential sources of variation in protective immunity within and between schistosome-infected populations are discussed in the following sections.

For filarial infections larvae-specific antibodies acquired with age have been shown to confer resistance to re-infection post-treatment (Day *et al*. 1991*b*); however, the immunogenic epitopes within the larval proteome could not be identified (Day *et al*. 1991*a*). Few studies have directly compared schistosome and nematode-specific antibody responses; however, studies to date suggest that differences do exist. *W. bancrofti* microfilaria and circulating antigen negative individuals were found to have the lowest parasite-specific IgG4:IgE (Jaoko *et al*. 2006) as has been observed in schistosome studies (Hagan *et al*. 1991). However, unlike in chronic schistosomiasis, the ratios did not differ between communities with high and low intensity infection indicating that relative changes in these isotypes may be less dependent on host exposure history (Jaoko *et al*. 2006). IgG2 also appears to limit infection intensity when directed against *W. bancrofti* microfilaria (Jaoko *et al*. 2006), but is ineffective against schistosome larvae (Demeure *et al*. 1993).

The specific target of antibody responses is particularly important for development of protective immunity, although the majority of field studies focus on crude antigen preparations. Early schistosome studies identified isotype-specific antibody responses to different antigen types within the parasite proteome (Langley *et al*. 1994), but it was not until the advent of mass spectrometric analysis that parasite peptides recognized by different antibody isotypes could be identified within crude preparations (Mutapi *et al*. 2005, 2008). Relative exposure of immunogenic peptides clearly plays a role in cumulative development of protective antibodies. For example, antibodies against the larval surface are associated with protection against *W. bancrofti* (Day *et al*. 1991*a*) and antibodies targeting cryptic antigens released by dying adult worms may influence the course of schistosome infection (Woolhouse and Hagan, 1999). It remains unclear whether there is a single antibody isotype that uniformly protects against helminth infection and it seems likely that redundancy within host antibody responses, variation between parasite species and/or host populations have all contributed to isotype-specific variations observed in the field.

Anti-pathology immune responses

Limiting immunopathology in patent helminth infection is a combination of parasite and host-mediated processes that synergize to limit aberrant immune reactivity and damage to the host. This is necessarily a compromise as dampening immune responses to infection also limits their efficacy at clearing infection (Maizels and Yazdanbakhsh, 2003). For tissue-dwelling helminths close association with host tissues throughout their life-cycle means that effector responses to adult worms and migrating larvae in these loci must be tightly regulated (Montenegro *et al*. 1999). In chronic schistosomiasis, for example, Th2-polarized responses account for the majority of host pathologies including hepatic fibrosis (Coutinho *et al*. 2007) and egg-specific responses promote granuloma formation (ElRidi *et al*. 1997), whilst Th2-mediated damage is mitigated by Th1-type (Henri *et al*. 2002; Dessein *et al*. 2004) and innate inflammatory cytokines (TNFα and IL-6 (Booth *et al*.

2004*a*; Wilson *et al*. 2008)). The importance of Tregs in balancing Th1 and Th17 responses in human filarial infections has been recently highlighted by Babu and colleagues who identified a positive correlation between lymphoedema and Th1/Th17 cytokine and Toll-like receptor expression in individuals with low expression of Treg-associated molecules (Babu *et al*. 2009).

Ubiquitous exposure to commensal bacteria and food antigens means that antigen-presentation and inflammatory responses are highly regulated in the gut to maintain a physical barrier between the lumen and host tissues (Mayer, 2000). GI nematodes are also able to actively down-regulate effector responses in the gut to maintain this regulatory environment (reviewed by Maizels and Yazdanbakhsh, 2003). Both *Ascaris* spp. and *Trichuris* spp. infections are also associated with reduced cellular responsiveness to both non-specific agonists and parasite-specific antigens in humans (Figueiredo *et al*. 2010). The rebound in *N. americanus*-specific IFNγ observed after anti-helminthic treatment to clear infection suggests that Th1 responses may be particularly regulated in chronic hookworm infection (Quinnell *et al*. 2004).

However, GI nematodes are not homogeneous in the immune responses that they induce and thus pose distinct immunopathological risks. *T. trichiura* infection induces a mixed Th1-Th2 cytokine profile (Faulkner *et al*. 2002), whilst *Ascaris lumbricoides* infection leads to a highly Th2 environment (Cooper *et al*. 2000). It has been postulated that murine *Trichuris* spp. are distinct from other GI infections in specifically up-regulating Th1 cytokines as a means of evading Th2-mediated clearance (refer to review by Else, 2005); however, fostering a more mixed cytokine response may also limit pathology. IL-10 secretion is highly prevalent in *T. trichiura* endemic populations with 97% of a Cameroonian cohort secreting parasite-specific IL-10 and older individuals producing the highest titres of non-specific IL-10 (Faulkner *et al*. 2002), suggesting that systemic immunoregulation coincides with cumulative exposure to infection.

SOURCES OF ACQUIRED IMMUNE HETEROGENEITY

Helminth life history

Helminths undergo complex life cycles leading to both physical and molecular variations during the course of an infection. Table 1 summarizes the key differences in helminth life histories that can introduce heterogeneity in the development of the host immune response to infection. Furthermore, the range of molecules and mechanisms that underlie helminth-mediated immunomodulation have been reviewed elsewhere (Maizels and Yazdanbakhsh, 2003; Maizels *et al*. 2009) and present a potential source of immune heterogeneity in themselves.

Parasite transmission route

Transmission route impacts upon where and how infection is initially detected by the immune system. Immature helminths face site-specific challenges as they invade their host, for example species that are transmitted through the skin tend to suffer immune attrition in this organ (He *et al*. 2005). Amongst the schistosome species, which invade by active penetration, *S. japonicum* cercariae migrate most rapidly to the dermis and this species also elicits the most pro-inflammatory response in human skin (He *et al*. 2002). In contrast *S. haematobium* and *S. mansoni* cercariae promote up-regulation of immunoregulatory proteins including IL-10 and IL-1 receptor antagonist (He *et al*. 2002), highlighting heterogeneity in the effector and regulatory immune mechanisms elicited even by closely-related species.

Arthropod-borne larvae are also exposed to immune attrition in human cutaneous tissue when they enter during blood-feeding by the vector. However, unlike actively penetrating parasites, antigens, enzymes and immunosuppressive molecules in arthropod saliva may skew the host immune response in favour of vector-transmitted larvae (Demeure *et al*. 2005). Furthermore, where a single helminth species (such as *W. bancrofti*) can be transmitted by multiple mosquito species of 4 different genera (*Aedes, Anopheles, Culex* and *Mansonia*) (Maizels and Kurniawan-Atmadja, 2002), there is potential for the human immune response to be differently shaped in each instance. The presence of *Wolbachia* spp. bacterial endosymbionts in mosquito vectors and almost all filarial helminth species has also been suggested to influence host T-cell responsiveness (Brattig, 2004), but this is yet to be verified.

On a broader scale transmission route can determine distribution of immune-mediated morbidities within host populations. One example is the lower prevalence of Sowda in *O. volvulus*-infected men relative to women inhabiting the same area, which has been attributed to development of immune tolerance after repeated exposure to *O. volvulus* vectors during agricultural work (Brattig, 2004; Trpis, 2006).

Following initial infection, larval migration may also contribute to immune heterogeneity between GI nematodes, particularly with respect to anti-pathology immunity. Clinical measures of atopic reactivity suggest that hookworm with lung-migratory stages effectively suppress asthma and wheeze in the lung (Scrivener *et al*. 2001; Leonardi-Bee *et al*. 2006). Therapeutic *N. americanus* infection is able to dampen inflammation both in the lung and in the gut, where adult worms reside (Falcone and Pritchard, 2005; Croese *et al*. 2006). On the other hand, *Trichuris* spp. which inhabit the lower intestine and

Table 1. Summary of parasite-factors that may influence the host acquired immune response to helminth infection (Anderson and May, 1992; Maizels *et al*. 1993; Maizels and Kurniawan-Atmadja, 2002; Gryseels *et al*. 2006; Hotez *et al*. 2008).

	Gastrointestinal (GI) nematodes				Filarial nematodes				*Schostosoma* spp. trematodes		
	A. lum	*E. ver*	*N. ame*	*Stro*. spp.	*T. tri*	*B. mal*	*O. vol*	*W. ban*	*S. hae*	*S. jap*	*S. man*
Distribution	Af, As, LAm	–	Af, As, LAm	Af, As, LAm	Af, As, LAm	SEAs	SSAf, LAm	As, SSAf, LAm	SSAf	China, SEAs	SSAf, Brazil
Human infections (millions)	807	–	576*	30–100**	604	–	37	120	207***	207***	207***
Intermediate host	None	None	None	None	None	*Ano* spp.	*Sim* spp.	*Ano, Cul Aed* spp.	*Bul* spp.	*Onc* spp.	*B. gla*
Transmission route	Faeco-oral	Faeco-oral	Skin penetration	Skin penetration	Faeco-oral	Vector	Vector	Vector	Skin penetration	Skin penetration	Skin penetration
Maturation delay	50–80 days	15–43 days	40–50 days	–	50–84 days	–	365 days	–	21–28 days	25–30 days	25–30 days
Adult worm life-span	1–2 years	< 1 year	2–3 years	–	1–2 years	–	8–10 years	3–5 years	3–5 years	3–5 years	6–11 years^
Adult worm length	15–35 cm	2–13 mm	7–11 mm	–	~ 4 cm	13–55 mm	19–50 mm	40–100 mm	7–20 mm	7–20 mm	7–20 mm
Adult worm niche	Ileum	Caecum	Ileum	Caecum	Caecum	Lymph	Skin	Lymph	Venous blood (bladder)	Venous blood (gut)	Venous blood (gut)
Fecundity per female	200,000 eggs/day	–	3000–6000 eggs/day	–	50–84 eggs/day	–	1000–2000 mf/day	–	3000 eggs/day	100–300 eggs/day	100–300 eggs/day

* All hookworm species.
** *Strongyloides stercoralis* only.
*** All *S. haematobium, S. japonicum* and *S. mansoni* infections.
^ Life-span of *S. mansoni* estimated using maximum likelihood techniques to assess pre- and post-treatment field data (Fulford *et al*. 1995).
Abbreviations: *A. lum* – *Ascaris lumbricoides, E. ver* – *Enterobis vermicularis, N. ame* – *Necator americanus, Stro* spp. – *Strongyloides* spp., *T. tri* – *Trichuris trichiura, B. mal* – *Brugia malayi, O. vol* – *Onchocerca volvulus, W. ban* – *Wuchereria bancrofti, S. hae* – *Schistosoma haematobium, S. jap* – *Schistosoma japonicum, S. man* – *Schistosoma mansoni*, Af – Africa, As – Asia, LAm – Latin America, SEAs – South East Asia, SSAf – Sub-Saharan Africa, *Ano* spp. – *Anopheles* spp. *mosquito, Aed* spp. – *Aedes* spp. *mosquito, Cul* spp. – *Culex* spp. *mosquito, B. gla* – *Biomphalaria glabrata* (*aquatic snail*), *Bul* spp. – *Bulinus* spp. (*aquatic snail*), mf – microfilariae.

do not migrate through the lung, can dampen inflammatory pathologies in the gut (Reddy and Fried, 2007) but are less effective at regulating clinical allergy elsewhere (Bager *et al*. 2010). Interestingly *A*. *lumbricoides* infection, which has a lung migratory phase, does not reduce asthmatic responses and is associated with elevated atopy (Flohr *et al*. 2009). There are many potential reasons for variation in the effect of GI helminth infection on clinical allergy (reviewed elsewhere (Flohr *et al*. 2009)) and conflicting results in existing literature (Leonardi-Bee *et al*. 2006) and a lack of studies which directly compare GI nematode infections with distinct migratory pathways make it difficult to conclude on the source of observed variations.

Life-cycle stages

Chronically infected individuals are simultaneously exposed to 3 helminth life-cycle stages; infective larvae, adult worms and transmission stage parasites (eggs, immature larvae or microfilariae). Both proteomic (Moreno and Geary, 2008) and DNA microarray studies (Jolly *et al*. 2007; Fitzpatrick *et al*. 2009) have shown that these life-cycle stages are molecularly different and thus elicit stage-specific immune responses that change over time.

In experimental murine studies where the course of infection can be tracked from its acute phase, there is a clear link between the onset of egg production (week 5–6 post-infection) and a Th1-to-Th2 shift in the cellular response to schistosomiasis (Pearce and MacDonald, 2002). Although the Th1-to-Th2 shift is less clear in human infections, defined egg peptides can specifically induce Th2 responses in human basophils, DCs and T cells *in vitro* (Schramm *et al*. 2003; Everts *et al*. 2009). Th2 polarization by schistosome eggs also induces granuloma formation (ElRidi *et al*. 1997), which facilitates passage of eggs from venous blood into the gut/bladder and subsequent transmission to the environment (Karanja *et al*. 1997; Pearce and MacDonald, 2002). Carbohydrate antigens on the schistosome egg surface also promote IgM secretion, which lacks immunological memory (Mutapi *et al*. 2003), whilst responses associated with protective immunity, such as IgE, IgG1 and IgG3, tend to emerge later in infection in response antigens released from dying adult worms (Woolhouse and Hagan, 1999).

There are notable distinctions between the type and magnitude of the cytokine response to egg-specific antigens and those directed against adult worm antigens (Joseph *et al*. 2004; Silveira *et al*. 2004). Adult *S*. *mansoni* worms tend to elicit a mixed Th1-Th2 cytokine profile (Williams *et al*. 1994) and are less effective at stimulating *in vitro* granuloma formation (IVGF) than egg antigens (ElRidi *et al*. 1997). Cross-sectional data from an *S*. *haematobium* endemic cohort suggests that adult worm-specific effector Th2 cytokines increase whilst IL-10 titres decline with age/exposure to infection (Mutapi *et al*. 2007) and thus effector responses to adult worms are higher in putatively resistant individuals.

In contrast to the Th2 cytokine responses induced by schistosome eggs and infective larvae of most nematodes, schistosome cercariae stimulate Th1/inflammatory mRNA expression in mice and resistance to larval invasion post-vaccination is dependent on IFNγ (Wynn *et al*. 1994). Although few studies have directly compared immune responses to the three schistosome life-cycle stages in humans, cercariae-specific IgM, IgG1 and IgG4 titres are higher than those directed against adult worm antigens (Viana *et al*. 1995). The distinct immune responses elicited by different helminth life-cycle stages underpin the development of so-called concomitant immunity, whereby *de novo* larval infection is blocked by adult worm-induced immunity but resident adult parasites are tolerated, a theory originally formulated for schistosome infections (Smithers and Terry, 1967). Studies have found evidence for development of PBMC-mediated resistance to L3 invasion in *O*. *volvulus* infection in an endemic area of Cameroon (MacDonald *et al*. 2002) and development of peripheral antibody-dependent immunity to *W*. *bancrofti* larvae with age in Papua New Guinea (Day *et al*. 1991*a*,*b*). There is little evidence for development of concomitant immunity to GI nematode infections; however, there is a distinct paucity of data in this area.

In addition to influencing anti-parasite immunity, helminth life-cycle stage contributes to heterogeneity in anti-pathology immune responses. Immunomodulatory processes can be life cycle stage-specific as characterized by distinct cytokine and proliferative responses to their respective antigens (Geiger *et al*. 2007). *In vitro* microfilariae are able to impair cytokine expression and maturation of human dendritic cells (DCs) (Semnani *et al*. 2001, 2004) and schistosome egg antigens can inhibit co-stimulatory molecule expression and skew APC cytokine secretion from an inflammatory to regulatory profile (Correale and Farez, 2009), with potential implications for the systemic CD4+ T cell phenotype. A variety of studies have also shown that cercariae excretory/secretory products actively modulate the host immune response during migration and maturation independent of egg or adult-worm-mediated processes (reviewed by Jenkins *et al*. 2005). However, whilst eggs, cercariae and microfilariae can impair and polarize host responses, the ability of parasites to evade and modulate immune recognition seems to increase as they mature (Nutman and Kumaraswami, 2001) and corresponds to large-scale switches in many suites of genes (Jolly *et al*. 2007) and active secretion of immunomodulatory molecules (Geiger *et al*. 2007). Thus, despite being exposed to the host

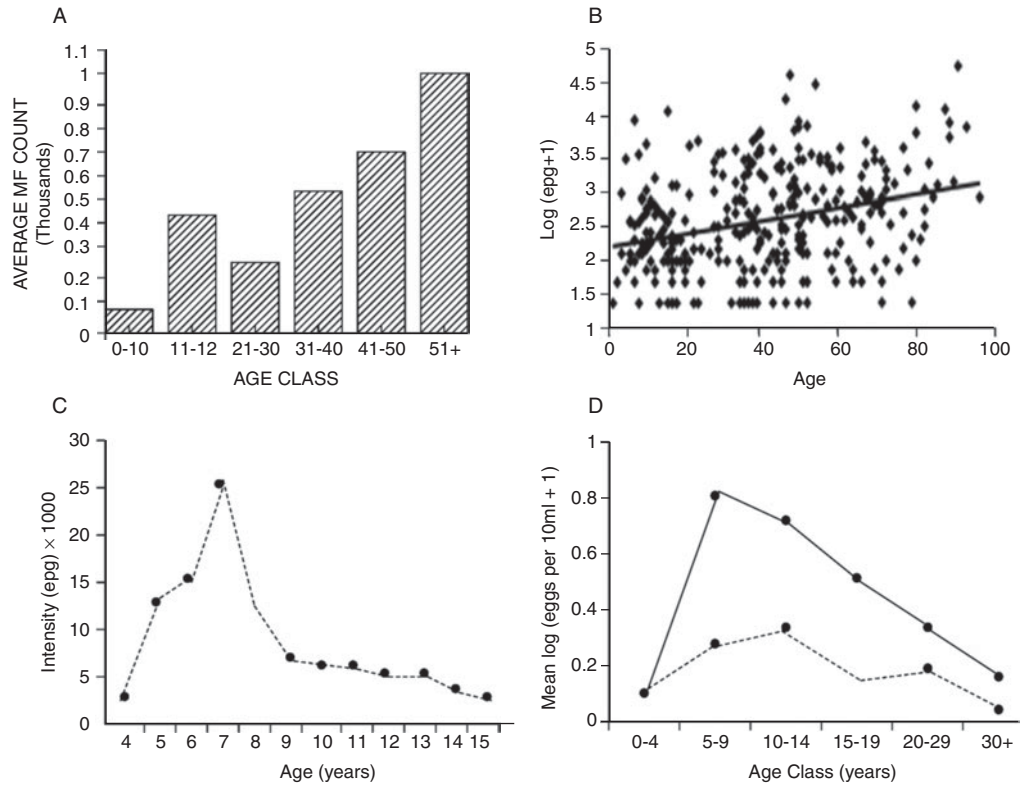

Fig. 2. The relationship between age and infection intensity in natural human helminthiases. (A) Mean *W. bancrofti* microfilaria (mf) count by age group ($n=156$, study area: Papua New Guinea, method: microscopy of 2 ml Giemsa-stained blood). Reprinted from the *American Journal of Tropical Medicine and Hygiene* (Day *et al*. 1991*b*), with permission from the managing editor. (B) Mean hookworm (*A. duodenale* and *N. americanus*) egg counts per gram faeces (EPG) by age group ($n=631$, study area: China, method: Kato-Katz thick smear). Reprinted from the *Journal of Parasitology* (Gandhi *et al*. 2001), with permission from Allen Press Publishing Services. (C) Mean *T. trichiuris* eggs per gram of faeces by age group ($n=96$, study area: Cameroon, method: Kato-Katz thick smear). Reprinted from the *Journal of Infectious Diseases* (Faulkner *et al*. 2002), with permission from the University of Chicago Press. (D) Age-infection intensity profiles of *S. haematobium* egg counts per 10 ml of urine from an area of low infection prevalence (dashed line) and an area of high infection prevalence (solid line) group ($n=133$ and 147, study area: Zimbabwe, method: filtration of 10 ml of urine) (re-drawn from Mutapi *et al*. 1997). Reprinted from *Parasitology Today* (Woolhouse, 1998), with permission from Elsevier and John Wiley and Sons Ltd.

immune system for the longest of all life-cycle stages, it is unsurprising that adult worms are so resistant to immune-mediated clearance (Maizels and Yazdanbakhsh, 2003), while immature parasites tend to be immunogenic, more readily killed by effector immune responses (Viana *et al*. 1995; Semnani *et al*. 2001; Medeiros *et al*. 2003) and are responsible for the majority of morbidity in helminth infections (Maizels and Yazdanbakhsh, 2003).

Parasite life-span

Unlike microbial infections which multiply exponentially in their hosts, helminths have evolved to invest in immune-evasive mechanisms to prolong intra-host survival and long-term fecundity (Jackson *et al*. 2009). Effective immune evasion and suppression by adult worms mean that reactivity to the antigens of live worms is limited (Geiger *et al*. 2007). Thus, hosts may only experience an immunogenic 'threshold' stimulus of parasite antigens once adult worms die (Mutapi *et al*. 2008) and resistance might be predicted to develop more slowly against long-lived worms than against shorter-lived species. The rapid switch to a protective immune profile following anti-helminthic drug treatment that kills adult worms is consistent with this hypothesis (Mutapi *et al*. 2003; Watanabe *et al*. 2007). If adult helminth life-span does cause a lag in the development of protective immunity then this may contribute to the variation in the age at which peak prevalence (Brooker *et al*. 2000) and infection intensity (Fig. 2) occurs in host populations exposed to different helminths.

Additionally, whilst some helminths have been known to survive for extremely long periods (Vermund *et al*. 1983), most die before reaching their maximum longevity. It is possible that the relatively short average helminth life-span in immunocompetent individuals reaps the maximal reproductive success relative to the physiological cost to the parasite of evading and modulating the host

immune response. In opposition to this, studies in hookworm suggest that the immune response has only a limited impact on parasite longevity (reviewed by Loukas and Prociv, 2001). Species with indirect life cycles, such as *Schistosoma* spp. and vector-borne helminths, tend to be long-lived (Table 1). Thus, once adult worms have adapted to their optimal host niche, they can produce offspring over a long period and, whilst they are immunogenic, adult worms do not appear to be the primary targets of the anti-helminth immune response in humans or in animal models (Smithers and Terry, 1967). This may be particularly true of *O. volvulus*, which has an average adult life-span of 8–10 years, but can have a maturation delay in the human host of over a year (Anderson and May, 1985). For short-lived worms, such as *E. vermicularis*, maturation and oviposition occurs much earlier, with an associated shift in stage-specific immune responses (Anderson and May, 1985).

GENETIC HETEROGENEITIES IN HELMINTH INFECTION

Host genetics

The human immune system has evolved in the context of helminth infection and this relationship has led to significant changes in our immune genes (Fumagalli *et al*. 2009; Maizels, 2009). A recent comprehensive study of 91 interleukin genes in 52 human populations found evidence for balancing selection in the evolution of the human immune response driven by helminth 'species richness', i.e. the greater diversity of helminths to which a population has been exposed, the greater diversity observed in interleukin alleles (Fumagalli *et al*. 2009). These findings are consistent with helminths differentially affecting the human acquired immune response at all levels of the CD4+ T cell axis, including gene families involved in inflammation in the skin, mucosal immunity, cell proliferation and survival and Th2 cytokines (Fumagalli *et al*. 2009). This is compounded by earlier studies indicating that many of the key genetic loci controlling the balance of effector and regulatory responses are polymorphic in the human population (Quinnell, 2003) and that certain human genes are associated with predisposition to infection with specific helminth parasites (Hoerauf *et al*. 2002; Peisong *et al*. 2004; Kouriba *et al*. 2005).

Additive genetic effects (heritability) significantly influence human infections with *A. lumbricoides* (Williams-Blangero *et al*. 2002*b*), *T. trichiura* (Williams-Blangero *et al*. 2002*a*), and *S. mansoni* (Bethony *et al*. 2002), whilst other studies have found host genetics to be less influential relative to exposure history (King *et al*. 2004). This conflicting evidence suggests that helminth distribution, allele frequency and host behaviour in each locality may determine the relative importance of heritable factors in patterns of infection and immunity (Ellis *et al*. 2007). Familial clustering of infection can further confound identification of genetic factors associated with helminth infection since *in utero* sensitization via transfer of helminth antigens and soluble immune factors from infected mothers to their offspring is independent of genotype (Eloi-Santos *et al*. 1989; Lammie *et al*. 1991; Novato-Silva *et al*. 1992) and co-habiting families often have similar exposure patterns (Smith *et al*. 2001). *T. trichiura* and *A. lumbricoides* for example were found to be significantly associated with shared living conditions; however, when infection distribution was investigated in more depth, only *T. trichiura* infection intensity was heritable (Ellis *et al*. 2007). In addition, populations in historically stable endemic areas may exhibit less heritable variation in host susceptibility if long-term selection against genotypes less favourable for a specific infection has occurred (King *et al*. 2004), particularly where other species of helminth infection are uncommon (Fumagalli *et al*. 2009).

Multiple gene loci have now been implicated in determining the degree of resistance to helminthiases and while many appear to relate to individual helminth species (reviewed by Quinnell, 2003), there are also loci controlling multiple susceptibilities, as is the case for IL-13 polymorphisms associated with predisposition to *S. haematobium* (Kouriba *et al*. 2005) and *O. volvulus* (Hoerauf *et al*. 2002).

Parasite genetics

Widespread genetic variation between parasite species is an equally important source of heterogeneity in the development of acquired immune responses to infection. Since parasitic nematodes have arisen from several independent evolutionary pathways their classification within the Phylum *Nematoda* belies the huge diversity apparent from their distinct life-histories, physiology and proteomes (Dorris *et al*. 1999). Comparisons between nematode worms at the genomic level have identified multiple species-specific gene sequences and transcription patterns, even between phylogenetically close organisms (Parkinson *et al*. 2004). Among the 3 major human *Schistosoma* spp. differences in immunologically relevant antigens have been found. Notably the leading anti-schistosome vaccine candidate antigen; glutathione-S-transferase (GST), of *S. mansoni* differs from that of *S. haematobium* (Trottein *et al*. 1992), though the impact of this on human immunity is unknown.

The existence of helminth homologues of human proteins (Pastrana *et al*. 1998; Gomez-Escobar *et al*. 2000) and analyses that indicate more rapid evolution among parasite secreted proteins are compatible with the idea that parasitic helminths are diversifying

fastest among antigens exposed to (and interacting with) the host immune system (Hoekstra *et al*. 2000; Harcus *et al*. 2004). The relatively recent completion of the first parasitic nematode (*B. malayi*; Ghedin *et al*. 2007) and trematode genome projects (*S. mansoni*; Berriman *et al*. 2009) and *S. japonicum* (Zhou *et al*. 2009) will inevitably yield greater insights into the evolution of genetic diversity between different helminth species.

Genetic diversity within helminth species may partly explain the slow development of protective immunity in endemic populations. It has been proposed that natural infections consist of several genetically distinct parasite strains (Galvani, 2005), evidence for which comes from variations in the non-coding sequence identified via genome-wide scans (Hunt *et al*. 2008; Redman *et al*. 2008). The effect of helminth strains on the host immune response has been investigated in theoretical models based on field studies of *A. lumbricoides, N. americanus, S. haematobium* and *T. trichiura* infections, which surmise that challenge with multiple strains delays development of resistance to infection in human populations because a different protective response must be mounted against each parasite genotype separately (Galvani, 2005).

INDIVIDUAL EXPOSURE HISTORY

The immune environment to which *de novo* helminth infections are exposed is not independent of a host's infection history (Woolhouse and Hagan, 1999). This was first demonstrated in a study of Sudanese canal workers hyper-exposed to *S. mansoni* infection, which showed that those who had been occupationally exposed for over 10 years were more resistant to infection than newly recruited workers (Satti *et al*. 1996). More recently a direct comparison between 2 occupationally exposed male cohorts, found that chronically exposed individuals develop resistance to *S. mansoni* re-infection after significantly fewer rounds of praziquantel treatment than those with a shorter exposure history (Black *et al*. 2010).

It is well known that in endemic populations worm burdens accumulate with age to a peak intensity and decline thereafter (Fisher, 1934) but the age at which peak prevalence occurs (Brooker *et al*. 2000) and the relative decline post-peak varies according to helminth species (Fig. 2). For example, intensity of *W. bancrofti* (Fig. 2A) and hookworm (Fig. 2B) infections tends to be highest in adults rather than children (Day *et al*. 1991*b*; Gandhi *et al*. 2001). In contrast, *T. trichiuria* (Fig. 2C) and schistosome (Fig. 2D) infection intensity peaks in childhood, after which worm burdens decline (Mutapi *et al*. 1997; Faulkner *et al*. 2002). Host behaviour may partially explain these differences, for example the risk of hookworm infection is highest in adults due to occupation-related exposure (Bradley and Chandiwana, 1990), whilst exposure to schistosomiasis is determined by contact with water from an early age during washing and domestic activities (Rudge *et al*. 2008).

Evidence that the host immune response is important in shaping age-related variation includes the observation that peak worm burdens occur at a younger age in regions of high infection intensity than in regions with low or intermediate intensity, a phenomenon called the 'peak shift' (Woolhouse, 1998) (Fig. 2D). It is clear that, at least for long-term residents of an area endemic for a particular species of helminth, age is effectively a proxy of exposure history and cumulative exposure to parasite antigens over time can trigger changes in the immune response (Mutapi *et al*. 2008). The latter assertion is supported by immuno-epidemiological studies in a variety of helminthiases indicating that cellular and humoral changes correlate with development of resistance to infection with age. For example in an *S. haematobium*-infected population, cross-sectional data showed that cytokine responses switch from a regulatory IL-10 response in younger individuals to an effector IL-5 response in older individuals (Mutapi *et al*. 2007). Longitudinal studies of *W. bancrofti*-infected subjects in Papua New Guinea showed that adults are relatively resistant to parasite accrual (Day *et al*. 1991*b*) and that this could be attributed to parallel age-specific acquisition of anti-larval antibodies (Day *et al*. 1991*a*). As discussed above, development of this form of concomitant immunity may be one explanation for the lower prevalence of these species in adults (Day *et al*. 1991*a*,*b*; MacDonald *et al*. 2002).

In areas of high intensity transmission, people are exposed to infection from a very early age and these patterns vary according to parasite species (Sousa-Figueiredo *et al*. 2008). A recent study in Zanzibar found that whilst schistosome infections were rare in pre-school children, soil-transmitted helminths were already highly prevalent (Sousa-Figueiredo *et al*. 2008). Variation in maternal infection status may also introduce variation in the *in utero* exposure patterns and potentially lead to long-lasting effects on the anti-helminth immune response of their offspring. For example, Steel and colleagues showed that pre-natal priming during maternal helminth infection has far-reaching effects on anti-filarial immunity including clonal deletion of parasite-specific T cells (Steel *et al*. 1994) and life-long susceptibility to infection (Steel and Nutman, 2003). Cellular studies in endemic populations have shown that umbilical cord blood lymphocytes (CBL) from neonates born of *W. bancrofti*-infected mothers mount parasite-specific cytokine responses similar to those of maternal PBMCs and in contrast to neonates born of un-infected mothers (Malhotra *et al*. 1997). An extension of this study found that schistosome and *B. malayi*-specific cytokine responses persisted in childhood and significantly affected the CD4+ T cell

polarization of *Bacillus* Calmette-Guerin (BCG) vaccine-specific responses in 2 to 10 year olds (Malhotra *et al*. 1999). However, an investigation of CBL responses to *N. americanus* and *O. volvulus* antigens found no evidence for specific polarization of Th1 and Th2 responses in the offspring of helminth-infected mothers (Pit *et al*. 2000). To date only a very few studies have directly compared the effect of different helminth species on neonatal immunity, though researchers hypothesize that tissue-dwelling helminths may be associated with a greater trans-placental transfer of antigens (Eloi-Santos *et al*. 1989; Novato-Silva *et al*. 1992; Malhotra *et al*. 1997). Since maternal exposure patterns will inevitably affect neonatal and early post-natal exposure the contribution of parasite transmission route and the associated behavioural risk factors for infection to immune heterogeneity (discussed above) should not be overlooked in early life.

ANTI-HELMINTHIC TREATMENT

Anti-helminthic treatment can effectively clear infection and thus artificially disrupts host-parasite immunoepidemiology, for example immune reactivity has been shown to peak shortly after treatment in many species of helminth infection (Quinnell *et al*. 2004; Watanabe *et al*. 2007). Following treatment, however, endemic populations become re-infected and the highest re-infection rates are consistently seen in individuals who carried high worm burdens before treatment suggesting pre-disposition to infection in certain hosts (Bundy *et al*. 1988; Tingley *et al*. 1988; Chandiwana *et al*. 1991).

In addition to clearing infection, even a single dose of chemotherapy can lead to changes in the host immune response to helminths. Successful treatment has been shown to enhance the proportion of effector T cells (Watanabe *et al*. 2007), increase the range of parasite proteins recognized by host antibodies and induce a more rapid switch to protective antibody isotypes (Mutapi *et al*. 2003) in schistosomiasis patients. This post-treatment rebound in immune responsiveness could be due to heightened DC activation in an environment of high parasite death (Watanabe *et al*. 2007) and/or recovery of normal immune function following removal of immunosuppressive parasite excretory/secretory products (Maizels and Yazdanbakhsh, 2003).

Furthermore, the large variation in treatment regimens employed in mass-treatment programmes, including the drug administered, number of treatments (single or repeated dose), age-ranges targeted (e.g. school-age children, whole population) and method of administration (e.g. school-based, hospital/clinic-based), is also a potential source of variation in host immunity within endemic populations. Notably, many treatment programmes exclude children under the age of 5 due to a perceived risk of side-effects, despite evidence that children can become both infected and a source of transmission from a very young age (Opara *et al*. 2007). Most also employ single-dose regimens, despite evidence that repeated treatment may be more effective at augmenting protective immunity (Black *et al*. 2010) potentially by periodically 'boosting' development of immunological memory (Woolhouse and Hagan, 1999). Whilst the long-term effects of anti-helminthic treatment on the host acquired immune response remain unclear, the desired reduction in parasite prevalence and transmission inevitably impacts upon helminth immunoepidemiology.

CO-INFECTION

Co-infection adds a further level of complexity to host-pathogen interactions and is highly prevalent in some communities, particularly in sub-Saharan Africa (Raso *et al*. 2004; Brooker *et al*. 2006). Here we focus on how co-infections of particular public health significance contribute to heterogeneity in the anti-helminth immune response and how these effects vary depending on the species of helminth infection.

Helminth-helminth co-infection

Certain combinations of helminth co-infection are more common in human populations than others, notably several nematode pairs including: *A. lumbricoides* and *T. trichiura* (Faulkner *et al*. 2005; Ellis *et al*. 2007), *A. lumbricoides* and hookworm (Fleming *et al*. 2006) and *O. volvulus* and *T. trichiura* (Faulkner *et al*. 2005). Interactions between schistosomes and nematodes in co-infection are more variable, with evidence for co-aggregation with hookworms (Fleming *et al*. 2006) and *T. trichiura* (Ellis *et al*. 2007), but no significant association with *A. lumbricoides* infection intensity (Tchuem Tchuente *et al*. 2003; Fleming *et al*. 2006; Ellis *et al*. 2007). Co-infection also tends to be associated with higher worm burdens than those seen in single species infections (Tchuem Tchuente *et al*. 2003). *A. lumbricoides-T. trichiura* co-infected individuals were also found to have higher IgG4:IgE ratios than their singly-infected counterparts (Figueiredo *et al*. 2010).

Several explanations for the variable effect of one helminth species on the likelihood of co-infection with another have been proposed, although it is unlikely that these factors act in isolation. Firstly, similarities in parasite life cycle might mean that certain species share common behavioural or genetic risk factors for infection (Ellis *et al*. 2007; Pullan *et al*. 2008). Alternatively, since studies in animals and humans show that patent infection with one helminth species can depress both the humoral and cellular responses of the host to challenge by other parasites

(Brady *et al*. 1999; Correa-Oliveira *et al*. 2002) it is possible that these synergistic interactions result from non-specific 'bystander suppression' (Brady *et al*. 1999) or immunosuppression directed against cross-reactive antigens expressed by closely-related species (Geiger *et al*. 2002). *S. mansoni*-exposed but patently uninfected Brazilian subjects, identified by egg and adult worm-specific antibodies, were found to have impaired responses to *Ascaris* spp. and hookworm infections (Correa-Oliveira *et al*. 2002), suggesting that prior exposure to schistosomiasis may have a lasting impact on host responses to other helminths. Experimental studies comparing *S. mansoni* infection with antigen (adult worm and egg homogenates) administration, suggest that immunosuppressive mechanisms rely on the presence of active infection (Osada and Kanazawa, 2010). However there is a lack of data on the effect of exposure to one parasite on the immunobiology of subsequent infection with a different species. It is conceivable that, even after clearance of adult worms, sequestration of eggs in host tissues, cellular memory responses to cross-reactive antigens and impaired organ-function resulting from cumulative morbidity may all affect the response to subsequent helminthiases.

Helminth-malaria co-infection

Malaria is a predominantly intracellular protozoan infection and has a markedly different immunobiology to that of helminths. The host response to malaria is characterized by inflammatory cytokines causing periodic fevers and immune-mediated pathology and, though protective immunity is yet to be fully characterized, clearance of blood-stage infection positively correlates with titres of variant-specific cytophilic antibodies, particularly IgG3 (Cavanagh *et al*. 2004).

Studies of the effect of malaria on immune responses to helminths are rare and tend to focus on the anti-malarial, rather than anti-helminth immune response. However, work on schistosomiasis-malaria co-infections indicate distinct effects on humoral and cellular responses (Mutapi *et al*. 2000; Wilson *et al*. 2008). On the one hand, co-infection has been associated with elevated titres of schistosome egg-specific IgE and IgG3 (Mutapi *et al*. 2000), indicating that acute inflammatory responses to malaria may reverse helminth-mediated immune hyporesponsiveness. Consistent with this hypothesis, skewing of T cell responses by malaria can exacerbate liver and spleen pathology due to deficient regulation of schistosome egg-specific regulatory responses (Wilson *et al*. 2008). Recent schistosome-malaria co-infection studies also show that co-infected children do not differ from singly infected children in their absolute numbers of circulating Tregs, but do exhibit reduced proportions of memory Tregs (Muok *et al*. 2009). Both elevated malaria-specific IgG3 and high *S. mansoni* egg counts are risk factors for splenomegaly (Booth *et al*. 2004*b*), suggesting that both severity of infection and host immunoreactivity contribute to the clinical outcome of malaria-schistosome co-infection (Diallo *et al*. 2004).

In individuals concurrently infected with GI nematodes and malaria, the inflammatory response to *Plasmodium* spp. might be expected to blunt Th2 effector responses involved in clearance of adult nematodes (Mountford and Pearlman, 1998). For example, *N. americanus*-specific Th2 effector cytokine and total IgE responses were lower in Papua New Guinean subjects co-infected with malaria, despite no observable effect on hookworm-specific IFNγ or cell proliferation (Quinnell *et al*. 2004). The same study found evidence for malaria and hookworm-mediated suppression of cell proliferation, but not effector cytokine secretion, in response to bacterial antigens (Quinnell *et al*. 2004). Thus, malaria co-infection can limit the effector response to hookworm, but may not significantly affect hookworm-mediated immunosuppressive mechanisms. Conversely, in a Malian study of malaria-*W. bancrofti* co-infection, filariasis was significantly associated with elevated total and malaria-specific IL-10 and reduced IFNγ (Metenou *et al*. 2009). As more co-infection studies are conducted it will become possible to more directly compare effects of malaria on the acquired immune responses mounted against different helminth species.

Helminth-HIV co-infection

Human Immunodeficiency Virus (HIV) is a lymphotrophic virus that replicates in CD4+ T cells leading to incremental abrogation of this cell population and progression to Acquired Immune Deficiency Syndrome (AIDS). Both HIV and helminth infection can be considered as immunocompromising infections, and it could therefore be predicted that these effects would synergise to the detriment of co-infected hosts.

Although co-infection studies tend to focus on immune responses to HIV, there is some evidence for heterogeneity in the reciprocal effects of HIV on helminth immunobiology. For example, HIV infection is associated with up-regulation of CTLA-4 expression and anergy, which might enhance helminth-mediated Treg induction and immune evasion (Steel and Nutman, 2003; Leng *et al*. 2006). Severe immunosuppression in advanced HIV/AIDS is also linked to abnormally high intensity *Strongyloides* spp. infections due to increased rates of auto-infection and, in some regions, strongyloidiasis is considered to be an AIDS-related opportunistic infection (Meamar *et al*. 2007). Furthermore, low CD4+ T cell counts in HIV seropositive individuals has been associated

with increased tissue sequestration of schistosome eggs (Karanja *et al*. 1997), although the relative impact that this has on host pathology has not been investigated.

In addition to generalized immunosuppression, HIV co-infections are negatively correlated with worm-specific effector cytokine responses (Sentongo *et al*. 1998) and may therefore reduce clearance of both adult and immature worms. HIV-positive patients co-infected with *S. mansoni* had lower measurable agonist-specific IL-4 and parasite and agonist-specific IL-10 than their HIV-negative counterparts, but had similar levels of IFNγ (Mwinzi *et al*. 2001). This finding was linked to an increased Th1: Th2 effector cytokine ratio in HIV-positive individuals (Mwinzi *et al*. 2001), which may result from preferential infection and abrogation of activated Th2 cells relative to Th1 and naïve T cells (Maggi *et al*. 1994).

Disruption of CD4+ T cell polarization and regulatory mechanisms might be predicted to exacerbate helminth-related pathologies (Maggi *et al*. 1994; Booth *et al*. 2004*a*), particularly in GI infections which require robust Th2 cytokine responses for clearance. However, very few studies of nematode-HIV co-infected populations have been conducted to date. HIV co-infection with schistosomiasis did not affect anti-helminthic treatment efficacy in a Kenyan cohort with high intensity *S. mansoni* infections (Karanja *et al*. 1998). Researchers in the latter study hypothesized that these findings were partly due to schistosomiasis preceding HIV infection and that subsequent co-infection did not affect pre-existing immune responses to schistosome antigens (Secor *et al*. 2004). The latter is of particular interest given that HIV is most prevalent in sexually-active adults and thus co-infections tend to arise after initial exposure to helminth infections, which peak in intensity in childhood. Thus, the stage of HIV infection (and associated degree of immunosuppression) and population-specific age-infection intensity distributions of different helminthiases are potential sources of heterogeneity and should be considered when investigating the immunobiology of co-infections.

CONCLUSIONS

Parasitic helminths present a diverse challenge to the immune system. Their large proteome and broad-range of antigens alone may partially explain the slow development of resistance to helminthiases (Yazdanbakhsh and Sacks, 2010). Furthermore, the stage-specific challenges (Day *et al*. 1991*a*,*b*; MacDonald *et al*. 2002) and genetic diversity within single species infections (Galvani, 2005) can lead to heterogeneity in anti-helminth immune responses in an individual host.

When comparing different species of helminth it is clear that immune-heterogeneity can transcend phylogenetic delineations, particularly with respect to parasite life-span and intra-host niche. Long-lived parasites and species inhabiting host tissues can only exist incognito via sophisticated immunosuppressive mechanisms that may compromise fecundity and transmission of infection (Karanja *et al*. 1997; Maizels and Yazdanbakhsh, 2003; Brattig, 2004). Short-lived and GI parasites face different challenges resulting in characteristic immunobiology. Whilst immunity to the tissue-dwelling nematodes is associated with a mixed CD4+ T cell immune response (Turaga *et al*. 2000), expulsion of GI nematode species is more specifically Th2-dependent (Turner *et al*. 2003). Few studies have directly compared immune responses to GI nematodes, filarial nematodes and *Schistosoma* spp. in single-infections, potentially due to the high prevalence of co-infection, meaning that many of the hypothesized sources of inter-specific immune heterogeneities remain to be empirically tested.

Unlike in experimental animal infections, acquired immune heterogeneity in natural infections also arises from host variables. Genetic polymorphism in the human immune system inevitably translates into heterogeneous expression of cellular and humoral responses, as is evident in clustering of immune responses within populations and individual families (Quinnell, 2003; Ellis *et al*. 2007). The relatively recent discovery of new CD4+ T cell subclasses and identification of further plasticity in innate effector cells has led to re-evaluation of how helminths interact with their human host (Diaz and Allen, 2007; Jenkins and Allen, 2010). Furthermore, the influence that host exposure-history, anti-helminthic treatment and co-infection have on anti-helminth responses indicates that immune heterogeneity arises not from the host and parasite in isolation, but also from the environment in which they interact.

Despite the diversity of helminth species and their host populations identifying the immunological mechanisms underlying these distinctions has been a challenge for field studies. Helminth-specific immune responses become activated and polarized at a very young age (Eloi-Santos *et al*. 1989; Lammie *et al*. 1991; Novato-Silva *et al*. 1992; Steel *et al*. 1994; King *et al*. 1998). However, field studies often exclude individuals under the age of 5, focusing instead on older individuals in the chronic stage of infection with a more ambiguous exposure and treatment history. In addition, whilst co-evolution of humans and helminths has clearly led to shared features of an anti-macroparasite response (Jackson *et al*. 2009), it is also possible that peripheral sampling methods, co-infection and long-term systemic disease may disguise integral differences in human immune responses (Hayes *et al*. 2004). The cellular targets of systemic and highly pleiotropic cytokines and site-specific immune responses are particularly difficult to

define in peripheral blood samples. Thus, in addition to expansion of elegant *in vitro* cellular assays, a wider range of immune correlates should be measured in the field to give a broader characterization of the host immune 'phenotype' in which these cells act.

Anthropophilic helminths are both evolutionarily ancient and alarmingly prevalent (Hotez *et al*. 2008), yet the immune responses mounted against them and the best means of treating infection remain unclear. Characterization of risk factors for infection and morbidity requires immunological measures to be considered in the context of host and environmental variables, which can be as influential as those of the parasite, and in some cases more so (Jackson *et al*. 2004*a*; Ellis *et al*. 2007).

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REFERENCES

Acosta, L. P., McManus, D. P., Aligui, G. D. L., Olveda, R. M. and Tiu, W. U. (2004). Antigen-specific antibody isotype patterns to *Schistosoma japonicum* recombinant and native antigens in a defined population in Leyte, The Philippines. *American Journal of Tropical Medicine and Hygiene* **70**, 549–555.

Anderson, R. M. and May, R. M. (1985). Helminth infections of humans – mathematical-models, population-dynamics, and control. *Advances in Parasitology* **24**, 1–101.

Anderson, R. M. and May, R. M. (1992). *Infectious Diseases of Humans. Dynamics and Control*, Oxford University Press, Oxford, UK.

Babu, S., Bhat, S. Q., Kumar, N. P., Lipira, A. P., Kumar, S., Karthick, C., Kumaraswami, V. and Nutman, T. B. (2009). Filarial lymphedema is characterized by antigen-specific Th1 and Th17 proinflammatory responses and a lack of regulatory T cells. *PLoS Neglected Tropical Diseases* **3**, 1–9.

Bager, P., Arnved, J., Ronborg, S., Wohlfahrt, J., Poulsen, L. K., Westergaard, T., Petersen, H. W., Kristensen, B., Thamsborg, S., Roepstorff, A., Kapel, C. and Melbye, M. (2010). *Trichuris suis* ova therapy for allergic rhinitis: A randomized, double-blind, placebo-controlled clinical trial. *Journal of Allergy and Clinical Immunology* **125**, 123–130.

Berriman, M., Haas, B. J., LoVerde, P. T., Wilson, R. A., Dillon, G. P., Cerqueira, G. C., Mashiyama, S. T., Al-Lazikani, B., Andrade, L. F., Ashton, P. D., Aslett, M. A., Bartholomeu, D. C., Blandin, G., Caffrey, C. R., Coghlan, A., Coulson, R., Day, T. A., Delcher, A., DeMarco, R., Djikeng, A., Eyre, T., Gamble, J. A., Ghedin, E., Gu, Y., Hertz-Fowler, C., Hirai, H., Hirai, Y., Houston, R., Ivens, A., Johnston, D. A., Lacerda, D., Macedo, C. D., McVeigh, P., Ning, Z. M., Oliveira, G., Overington, J. P., Parkhill, J., Pertea, M., Pierce, R. J., Protasio, A. V., Quail, M. A., Rajandream, M. A., Rogers, J., Sajid, M., Salzberg, S. L., Stanke, M., Tivey, A. R., White, O., Williams, D. L., Wortman, J., Wu, W. J., Zamanian, M., Zerlotini, A., Fraser-Liggett, C. M., Barrell, B. G. and El-Sayed, N. M. (2009). The genome of the blood fluke *Schistosoma mansoni*. *Nature, London* **460**, 352–365.

Bethony, J., Williams, J. T., Blangero, J., Kloos, H., Gazzinelli, A., Soares, B., Coelho, L., Alves-Fraga, L., Williams-Blangero, S., Loverde, P. T. and Correa-Oliveira, R. (2002). Additive host genetic factors influence fecal egg excretion rates during *Schistosoma mansoni* infection in a rural area in Brazil. *American Journal of Tropical Medicine and Hygiene* **67**, 336–343.

Black, C. L., Mwinzi, P. N., Muok, E. M., Abudho, B., Fitzsimmons, C. M., Dunne, D. W., Karanja, D. M., Secor, W. E. and Colley, D. G. (2010). Influence of exposure history on the immunology and development of resistance to human Schistosomiasis mansoni. *PLoS Neglected Tropical Diseases* **4**, e637.

Booth, M., Mwatha, J. K., Joseph, S., Jones, F. M., Kadzo, H., Ireri, E., Kazibwe, F., Kemijumbi, J., Kariuki, C., Kimani, G., Ouma, J. H., Kabatereine, N. B., Vennervald, B. J. and Dunne, D. W. (2004*a*). Periportal fibrosis in human *Schistosoma mansoni* infection is associated with low IL-10, low IFN-gamma, high TNF-alpha, or low RANTES, depending on age and gender. *Journal of Immunology* **172**, 1295–1303.

Booth, M., Vennervald, B. J., Kenty, L., Butterworth, A. E., Kariuki, H. C., Kadzo, H., Ireri, E., Amaganga, C., Kimani, G., Mwatha, J. K., Otedo, A., Ouma, J. H., Muchiri, E. and Dunne, D. W. (2004*b*). Micro-geographical variation in exposure to *Schistosoma mansoni* and malaria, and exacerbation of splenomegaly in Kenyan school-aged children. *BMC Infectious Diseases* **4**, 13.

Bradley, M. and Chandiwana, S. K. (1990). Age-dependency in predisposition to hookworm infection in the Burma valley area of Zimbabwe. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 826–828.

Brady, M. T., O'Neill, S. M., Dalton, J. P. and Mills, K. H. G. (1999). *Fasciola hepatica* suppresses a protective Th1 response against *Bordetella pertussis*. *Infection and Immunity* **67**, 5372–5378.

Brattig, N. W. (2004). Pathogenesis and host responses in human onchocerciasis: impact of *Onchocerca* filariae and *Wolbachia* endobacteria. *Microbes and Infection* **6**, 113–128.

Brooker, S., Clements, A. C., Hotez, P. J., Hay, S. I., Tatem, A. J., Bundy, D. A. and Snow, R. W. (2006). The co-distribution of *Plasmodium falciparum* and

hookworm among African schoolchildren. *Malaria Journal* **5**, 99.

Brooker, S., Donnelly, C. A. and Guyatt, H. L. (2000). Estimating the number of helminthic infections in the Republic of Cameroon from data on infection prevalence in schoolchildren. *Bulletin of the World Health Organization* **78**, 1456–1465.

Bundy, D. A., Cooper, E. S., Thompson, D. E., Didier, J. M. and Simmons, I. (1988). Effect of age and initial infection intensity on the rate of reinfection with *Trichuris trichiura* after treatment. *Parasitology* **97**, 469–476.

Caldas, I. R., Correa-Oliveira, R., Colosimo, E., Carvalho, O. S., Massara, C. L., Colley, D. G. and Gazzinelli, G. (2000). Susceptibility and resistance to *Schistosoma mansoni* reinfection: Parallel cellular and isotypic immunologic assessment. *American Journal of Tropical Medicine and Hygiene* **62**, 57–64.

Cavanagh, D. R., Dodoo, D., Hviid, L., Kurtzhals, J. A., Theander, T. G., Akanmori, B. D., Polley, S., Conway, D. J., Koram, K. and McBride, J. S. (2004). Antibodies to the N-terminal block 2 of *Plasmodium falciparum* merozoite surface protein 1 are associated with protection against clinical malaria. *Infection and Immunity* **72**, 6492–6502.

Chandiwana, S. K., Woolhouse, M. E. J. and Bradley, M. (1991). Factors affecting the intensity of reinfection with *Schistosoma haematobium* following treatment with praziquantel. *Parasitology* **102**, 73–83.

Cooper, P. J., Chico, M. E., Sandoval, C., Espinel, I., Guevara, A., Kennedy, M. W., Urban Jr, J. F., Griffin, G. E. and Nutman, T. B. (2000). Human infection with *Ascaris lumbricoides* is associated with a polarized cytokine response. *Journal of Infectious Diseases* **182**, 1207–1213.

Cooper, P. J., Chico, M. E., Sandoval, C. and Nutman, T. B. (2004). Atopic phenotype is an important determinant of immunoglobulin E-mediated inflammation and expression of T helper cell type 2 cytokines to ascaris antigens in children exposed to *Ascariasis*. *Journal of Infectious Diseases* **190**, 1338–1346.

Correa-Oliveira, R., Golgher, D. B., Oliveira, G. C., Carvalho, O. S., Massara, C. L., Caldas, I. R., Colley, D. G. and Gazzinelli, G. (2002). Infection with S*chistosoma mansoni* correlates with altered immune responses to *Ascaris lumbricoides* and hookworm. *Acta Tropica* **83**, 123–132.

Correale, J. and Farez, M. (2009). Helminth antigens modulate immune responses in cells from multiple sclerosis patients through TLR2-dependent mechanisms. *Journal of Immunology* **183**, 5999–6012.

Coutinho, H. M., Acosta, L. P., Wu, H. W., McGarvey, S. T., Su, L., Langdon, G. C., Jiz, M. A., Jarilla, B., Olveda, R. M., Friedman, J. F. and Kurtis, J. D. (2007). Th2 cytokines are associated with persistent hepatic fibrosis in human *Schistosoma japonicum* infection. *Journal of Infectious Diseases* **195**, 288–295.

Croese, J., O'Neil, J., Masson, J., Cooke, S., Melrose, W., Pritchard, D. and Speare, R. (2006). A proof of concept study establishing *Necator americanus* in Crohn's patients and reservoir donors. *Gut* **55**, 136–137.

Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R. A., Mitsdoerffer, M., Strom, T. B., Elyaman, W., Ho, I. C., Khoury, S., Oukka, M. and Kuchroo, V. K. (2008). IL-4 inhibits TGF-beta-induced Foxp3(+) T cells and, together with TGF-beta, generates IL-9(+) IL-10(+) Foxp3(-) effector T cells. *Nature Immunology* **9**, 1347–1355.

Day, K. P., Gregory, W. F. and Maizels, R. M. (1991*a*). Age-specific acquisition of immunity to infective larvae in a bancroftian filariasis endemic Area of Papua-New-Guinea. *Parasite Immunology* **13**, 277–290.

Day, K. P., Grenfell, B., Spark, R., Kazura, J. W. and Alpers, M. P. (1991*b*). Age-specific patterns of change in the dynamics of *Wuchereria bancrofti* infection in Papua-New-Guinea. *American Journal of Tropical Medicine and Hygiene* **44**, 518–527.

De Jesus, A. R., Araujo, I., Bacellar, O., Magalhaes, A., Pearce, E., Harn, D., Strand, M. and Carvalho, E. M. (2000). Human immune responses to *Schistosoma mansoni* vaccine candidate antigens. *Infection and Immunity* **68**, 2797–2803.

Deenick, E. K. and Tangye, S. G. (2007). Autoimmunity: IL-21: a new player in Th17-cell differentiation. *Immunology and Cell Biology* **85**, 503–505.

Demeure, C. E., Brahimi, K., Hacini, F., Marchand, F., Peronet, R., Huerre, M., St-Mezard, P., Nicolas, J. F., Brey, P., Delespesse, G. and Mecheri, S. (2005). *Anopheles* mosquito bites activate cutaneous mast cells leading to a local inflammatory response and lymph node hyperplasia. *Journal of Immunology* **174**, 3932–3940.

Demeure, C. E., Rihet, P., Abel, L., Ouattara, M., Bourgois, A. and Dessein, A. J. (1993). Resistance to *Schistosoma mansoni* in humans - influence of the IgE/IgG4 balance and IgG2 in immunity to reinfection after chemotherapy. *Journal of Infectious Diseases* **168**, 1000–1008.

Dessein, A., Kouriba, B., Eboumbou, C., Dessein, H., Argiro, L., Marquet, S., Elwali, N. E. M. A., Rodrigues, V., Li, Y. S., Doumbo, O. and Chevillard, C. (2004). Interleukin-13 in the skin and interferon-gamma in the liver are key players in immune protection in human schistosomiasis. *Immunological Reviews* **201**, 180–190.

Diallo, T. O., Remoue, F., Schacht, A. M., Charrier, N., Dompnier, J. P., Pillet, S., Garraud, O., N'Diaye, A. A., Capron, A., Capron, M. and Riveau, G. (2004). Schistosomiasis co-infection in humans influences inflammatory markers in uncomplicated *Plasmodium falciparum* malaria. *Parasite Immunology* **26**, 365–369.

Diaz, A. and Allen, J. E. (2007). Mapping immune response profiles: The emerging scenario from helminth immunology. *European Journal of Immunology* **37**, 3319–3326.

Doetze, A., Satoguina, J., Burchard, G., Rau, T., Loliger, C., Fleischer, B. and Hoerauf, A. (2000). Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by Th3/Tr1-type cytokines IL-10 and transforming growth factor-beta but not by a Th1 to Th2 shift. *International Immunology* **12**, 623–630.

Dorris, M., De Ley, P. and Blaxter, M. L. (1999). Molecular analysis of nematode diversity and the evolution of parasitism. *Parasitology Today* **15**, 188–193.

Ellis, M. K., Raso, G., Li, Y. S., Rong, Z., Chen, H. G. and McManus, D. P. (2007). Familial aggregation of human susceptibility to co- and multiple helminth infections in a population from the Poyang Lake region, China. *International Journal for Parasitology* **37**, 1153–1161.

Eloi-Santos, S. M., Novato-Silva, E., Maselli, V. M., Gazzinelli, G., Colley, D. G. and Correa-Oliveira, R. (1989). Idiotypic sensitization in utero of children born to mothers with schistosomiasis or Chagas' disease. *Journal of Clinical Investigation* **84**, 1028–1031.

ElRidi, R., Ismail, S., Gaafar, T. and ElDemellawy, M. (1997). Differential responsiveness of humans with early-stage schistosomiasis haematobium to *Schistosoma haematobium* soluble adult-worm and egg antigens. *Parasitology Research* **83**, 471–477.

Else, K. J. (2005). Have gastrointestinal nematodes outwitted the immune system? *Parasite Immunology* **27**, 407–415.

Everts, B., Perona-Wright, G., Smits, H. H., Hokke, C. H., van der Ham, A. J., Fitzsimmons, C. M., Doenhoff, M. J., van der Bosch, J., Mohrs, K., Haas, H., Mohrs, M., Yazdanbakhsh, M. and Schramm, G. (2009). Omega-1, a glycoprotein secreted by *Schistosoma mansoni* eggs, drives Th2 responses. *Journal of Experimental Medicine* **206**, 1673–1680.

Falcone, F. H. and Pritchard, D. I. (2005). Parasite role reversal: worms on trial. *Trends in Parasitology* **21**, 157–160.

Faulkner, H., Turner, J., Behnke, J., Kamgno, J., Rowlinson, M. C., Bradley, J. E. and Boussinesq, M. (2005). Associations between filarial and gastrointestinal nematodes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99**, 301–312.

Faulkner, H., Turner, J., Kamgno, J., Pion, S. D., Boussinesq, M. and Bradley, J. E. (2002). Age- and infection intensity-dependent cytokine and antibody production in human trichuriasis: the importance of IgE. *Journal of Infectious Diseases* **185**, 665–672.

Figueiredo, C. A., Barreto, M. L., Rodrigues, L. C., Cooper, P. J., Silva, N. B., Amorim, L. D. and Alcantara-Neves, N. M. (2010). Chronic intestinal helminth infections are associated with immune hyporesponsiveness and induction of a regulatory network. *Infection and Immunology* **78**, 3160–3167.

Finkelman, F. D., Shea-Donohue, T., Morris, S. C., Gildea, L., Strait, R., Madden, K. B., Schopf, L. and Urban, J. F. (2004). Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunological Reviews* **201**, 139–155.

Fisher, A. C. (1934). A study of the schistosomiasis of the Stanleyville district of the Belgian congo. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **28**, 277–306.

Fitzpatrick, J. M., Peak, E., Perally, S., Chalmers, I. W., Barrett, J., Yoshino, T. P., Ivens, A. C. and Hoffmann, K. F. (2009). Anti-schistosomal intervention targets identified by lifecycle transcriptomic analyses. *PLoS Neglected Tropical Diseases* **3**, 543.

Fleming, F. M., Brooker, S., Geiger, S. M., Caldas, I. R., Correa-Oliveira, R., Hotez, P. J. and Bethony, J. M. (2006). Synergistic associations between hookworm and other helminth species in a rural community in Brazil. *Tropical Medicine & International Health* **11**, 56–64.

Flohr, C., Quinnell, R. J. and Britton, J. (2009). Do helminth parasites protect against atopy and allergic disease? *Clinical and Experimental Allergy* **39**, 20–32.

Fulford, A. J. C., Butterworth, A. E., Ouma, J. H. and Sturrock, R. F. (1995). A statistical approach to schistosome population dynamics and estimation of the life-span of *Schistosoma mansoni* in man. *Parasitology* **110**, 307–316.

Fumagalli, M., Pozzoli, U., Cagliani, R., Comi, G. P., Riva, S., Clerici, M., Bresolin, N. and Sironi, M. (2009). Parasites represent a major selective force for interleukin genes and shape the genetic predisposition to autoimmune conditions. *Journal of Experimental Medicine* **206**, 1395–1408. doi: 10.1084/jem.20082779.

Galvani, A. P. (2005). Age-dependent epidemiological patterns and strain diversity in helminth parasites. *Journal of Parasitology* **91**, 24–30.

Gandhi, N. S., Chen, J. Z., Khoshnood, K., Xing, F. Y., Li, S. W., Liu, Y. R., Zhan, B., Xue, H. C., Tong, C. J., Wang, Y., Wang, W. S., He, D. X., Chen, C., Xiao, S. H., Hawdon, J. M. and Hotez, P. J. (2001). Epidemiology of *Necator americanus* hookworm infections in Xiulongkan village, Hainan province, China: High prevalence and intensity among middle-aged and elderly residents. *Journal of Parasitology* **87**, 739–743.

Geiger, S. M., Caldas, I. R., Mc Glone, B. E., Campi-Azevedo, A. C., De Oliveira, L. M., Brooker, S., Diemert, D., Correa-Oliveira, R. and Bethony, J. M. (2007). Stage-specific immune responses in human *Necator americanus* infection. *Parasite Immunology* **29**, 347–358.

Geiger, S. M., Massara, C. L., Bethony, J., Soboslay, P. T., Carvalho, O. S. and Correa-Oliveira, R. (2002). Cellular responses and cytokine profiles in *Ascaris lumbricoides* and *Trichuris trichiura* infected patients. *Parasite Immunology* **24**, 499–509.

Ghedin, E., Wang, S. L., Spiro, D., Caler, E., Zhao, Q., Crabtree, J., Allen, J. E., Delcher, A. L., Guiliano, D. B., Miranda-Saavedra, D., Angiuoli, S. V., Creasy, T., Amedeo, P., Haas, B., El-Sayed, N. M., Wortman, J. R., Feldblyum, T., Tallon, L., Schatz, M., Shumway, M., Koo, H., Salzberg, S. L., Schobel, S., Pertea, M., Pop, M., White, O., Barton, G. J., Carlow, C. K. S., Crawford, M. J., Daub, J., Dimmic, M. W., Estes, C. F., Foster, J. M., Ganatra, M., Gregory, W. F., Johnson, N. M., Jin, J. M., Komuniecki, R., Korf, I., Kumar, S., Laney, S., Li, B. W., Li, W., Lindblom, T. H., Lustigman, S., Ma, D., Maina, C. V., Martin, D. M. A., McCarter, J. P., McReynolds, L., Mitreva, M., Nutman, T. B., Parkinson, J., Peregrin-Alvarez, J. M., Poole, C., Ren, Q. H., Saunders, L., Sluder, A. E., Smith, K., Stanke, M., Unnasch, T. R.,

Ware, J., Wei, A. D., Weil, G., Williams, D. J., Zhang, Y. H., Fraser-Liggett, C., Slatko, B., Blaxter, M. L. and Scott, A. L. (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. *Science* **317**, 1756–1760.

Gomez-Escobar, N., Gregory, W. F. and Maizels, R. M. (2000). Identification of tgh-2, a filarial nematode homolog of *Caenorhabditis elegans* daf-7 and human transforming growth factor beta, expressed in microfilarial and adult stages of *Brugia malayi*. *Infection and Immunity* **68**, 6402–6410.

Gryseels, B., Polman, K., Clerinx, J. and Kestens, L. (2006). Human schistosomiasis. *Lancet* **368**, 1106–1118.

Hagan, P., Blumenthal, U. J., Dunn, D., Simpson, A. J. G. and Wilkins, H. A. (1991). Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. *Nature, London* **349**, 243–245.

Harcus, Y. M., Parkinson, J., Fernandez, C., Daub, J., Selkirk, M. E., Blaxter, M. L. and Maizels, R. M. (2004). Signal sequence analysis of expressed sequence tags from the nematode *Nippostrongylus brasiliensis* and the evolution of secreted proteins in parasites. *Genome Biology* **5**, R39.

Hayes, K. S., Bancroft, A. J. and Grencis, R. K. (2004). Immune-mediated regulation of chronic intestinal nematode infection. *Immunological Reviews* **201**, 75–88.

He, Y. X., Chen, L. and Ramaswamy, K. (2002). *Schistosoma mansoni, S. haematobium*, and *S. japonicum*: early events associated with penetration and migration of schistosomula through human skin. *Experimental Parasitology* **102**, 99–108.

He, Y. X., Salafsky, B. and Ramaswamy, K. (2005). Comparison of skin invasion among three major species of *Schistosoma*. *Trends in Parasitology* **21**, 201–203.

Henri, S., Chevillard, C., Mergani, A., Paris, P., Gaudart, J., Camilla, C., Dessein, H., Montero, F., Elwali, N. E., Saeed, O. K., Magzoub, M. and Dessein, A. J. (2002). Cytokine regulation of periportal fibrosis in humans infected with *Schistosoma mansoni*: IFN-gamma is associated with protection against fibrosis and TNF-alpha with aggravation of disease. *Journal of Immunology* **169**, 929–936.

Hoekstra, R., Visser, A., Otsen, M., Tibben, J., Lenstra, J. A. and Roos, M. H. (2000). EST sequencing of the parasitic nematode *Haemonchus contortus* suggests a shift in gene expression during transition to the parasitic stages. *Molecular and Biochemical Parasitology* **110**, 53–68.

Hoerauf, A., Kruse, S., Brattig, N. W., Heinzmann, A., Mueller-Myhsok, B. and Deichmann, K. A. (2002). The variant Arg110Gln of human IL-13 is associated with an immunologically hyper-reactive form of onchocerciasis (sowda). *Microbes and Infection* **4**, 37–42.

Hoffmann, K. F., Wynn, T. A. and Dunne, D. W. (2002). Cytokine-mediated host responses during schistosome infections; walking the fine line between immunological control and immunopathology. *Advances in Parasitology* **52**, 265–307.

Hotez, P. J., Brindley, P. J., Bethony, J. M., King, C. H., Pearce, E. J. and Jacobson, J. (2008). Helminth infections: the great neglected tropical diseases. *Journal of Clinical Investigation* **118**, 1311–1321.

Hunt, P. W., Knox, M. R., Le Jambre, L. F., McNally, J. and Anderson, L. J. (2008). Genetic and phenotypic differences between isolates of *Haemonchus contortus* in Australia. *International Journal for Parasitology* **38**, 885–900.

Jackson, J. A., Friberg, I. M., Little, S. and Bradley, J. E. (2009). Review series on helminths, immune modulation and the hygiene hypothesis: immunity against helminths and immunological phenomena in modern human populations: coevolutionary legacies? *Immunology* **126**, 18–27.

Jackson, J. A., Turner, J. D., Rentoul, L., Faulkner, H., Behnke, J. M., Hoyle, A., Grencis, R. K., Else, K. J., Kamgno, J., Bradley, J. E. and Boussinesq, M. (2004*a*). Cytokine response profiles predict species-specific infection patterns in human GI nematodes. *International Journal for Parasitology* **34**, 1237–1244.

Jackson, J. A., Turner, J. D., Rentoul, L., Faulkner, H., Behnke, J. M., Hoyle, M., Grencis, R. K., Else, K. J., Kamgno, J., Boussinesq, M. and Bradley, J. E. (2004*b*). T helper cell type 2 responsiveness predicts future susceptibility to gastrointestinal nematodes in humans. *Journal of Infectious Diseases* **190**, 1804–1811.

Jaoko, W. G., Simonsen, P. E., Meyrowitsch, D. W., Estambale, B. B. A., Malecela-Lazaro, M. N. and Michael, E. (2006). Filarial-specific antibody response in east African Bancroftian filariasis: Effects of host infection, clinical disease, and filarial endemicity. *American Journal of Tropical Medicine and Hygiene* **75**, 97–107.

Jenkins, S. J. and Allen, J. E. (2010). Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes. *Journal of Biomedicine and Biotechnology* **2010**, 262609.

Jenkins, S. J., Hewitson, J. P., Jenkins, G. R. and Mountford, A. P. (2005). Modulation of the host's immune response by schistosome larvae. *Parasite Immunology* **27**, 385–393.

Jolly, E. R., Chin, C. S., Miller, S., Bahgat, M. M., Lim, K. C., DeRisi, J. and McKerrow, J. H. (2007). Gene expression patterns during adaptation of a helminth parasite to different environmental niches. *Genome Biology* **8**, R65.

Joseph, S., Jones, F. M., Kimani, G., Mwatha, J. K., Kamau, T., Kazibwe, F., Kemijumbi, J., Kabatereine, N. B., Booth, M., Kariuki, H. C., Ouma, J. H., Vennervald, B. J. and Dunne, D. W. (2004). Cytokine production in whole blood cultures from a fishing community in an area of high endemicity for *Schistosoma mansoni* in Uganda: the differential effect of parasite worm and egg antigens. *Infection and Immunity* **72**, 728–734.

Karanja, D. H. S., Boyer, A. E., Strand, M., Colley, D. G., Nahlen, B. L., Ouma, J. H. and Secor, W. E. (1998). Studies on schistosomiasis in western Kenya: II. Efficacy of praziquantel for treatment of schistosomiasis in persons coinfected with human immunodeficiency virus-1. *American Journal of Tropical Medicine and Hygiene* **59**, 307–311.

Karanja, D. M. S., Colley, D. G., Nahlen, B. L., Ouma, J. H. and Secor, W. E. (1997). Studies on schistosomiasis in western Kenya .I. Evidence for immune-facilitated excretion of schistosome eggs from patients with *Schistosoma mansoni* and human immunodeficiency virus coinfections. *American Journal of Tropical Medicine and Hygiene* **56**, 515–521.

King, C. H., Blanton, R. E., Muchiri, E. M., Ouma, J. H., Kariuki, H. C., Mungai, P., Magak, P., Kadzo, H., Ireri, E. and Koech, D. K. (2004). Low heritable component of risk for infection intensity and infection-associated disease in urinary schistosomiasis among Wadigo village populations in Coast Province, Kenya. *American Journal of Tropical Medicine and Hygiene* **70**, 57–62.

King, C. L., Malhotra, I., Mungai, P., Wamachi, A., Kioko, J., Ouma, J. H. and Kazura, J. W. (1998). Cell sensitization to helminthic infection develops *in utero* in humans. *Journal of Immunology* **160**, 3578–3584.

Klion, A. D. and Nutman, T. B. (2004). The role of eosinophils in host defense against helminth parasites. *Journal of Allergy and Clinical Immunology* **113**, 30–37.

Kouriba, B., Chevillard, C., Bream, J. H., Argiro, L., Dessein, H., Arnaud, V., Sangare, L., Dabo, A., Beavogui, A. H., Arama, C., Traore, H. A., Doumbo, O. and Dessein, A. (2005). Analysis of the 5q31-q33 locus shows an association between IL13-1055C/T IL-13-591A/G polymorphisms and *Schistosoma haematobium* infections. *Journal of Immunology* **174**, 6274–6281.

Kurowska-Stolarska, M., Kewin, P., Murphy, G., Russo, R. C., Stolarski, B., Garcia, C. C., Komai-Koma, M., Pitman, N., Li, Y. B., McKenzie, A. N. J., Teixeira, M. M., Liew, F. Y. and Xu, D. M. (2008). IL-33 induces antigen-specific IL-5(+) T cells and promotes allergic-induced airway inflammation independent of IL-4. *Journal of Immunology* **181**, 4780–4790.

Lammie, P. J., Hitch, W. L., Allen, E. M. W., Hightower, W. and Eberhard, M. L. (1991). Maternal filarial infection as risk factor for infection in children. *Lancet* **337**, 1005–1006.

Langley, J. G., Kariuki, H. C., Hammersley, A. P., Ouma, J. H., Butterworth, A. E. and Dunne, D. W. (1994). Human-IgG subclass responses and subclass restriction to *Schistosoma mansoni* egg antigens. *Immunology* **83**, 651–658.

Leng, Q. B., Bentwich, Z. and Borkow, G. (2006). Increased TGF-beta, Cbl-b and CTLA-4 levels and immunosuppression in association with chronic immune activation. *International Immunology* **18**, 637–644.

Leonardi-Bee, J., Pritchard, D., Britton, J. and Collaboration, P. A. (2006). Asthma and current intestinal parasite infection – Systematic review and meta-analysis. *American Journal of Respiratory and Critical Care Medicine* **174**, 514–523.

Loukas, A. and Prociv, P. (2001). Immune responses in hookworm infections. *Clinical Microbiology Reviews* **14**, 689–703.

MacDonald, A. J., Turaga, P. S. D., Harmon-Brown, C., Tierney, T. J., Bennett, K. E., McCarthy, M. C., Simonek, S. C., Enyong, P. A., Moukatte, D. W. and Lustigman, S. (2002). Differential cytokine and antibody responses to adult and larval stages of *Onchocerca volvulus* consistent with the development of concomitant immunity. *Infection and Immunity* **70**, 2796–2804.

Maggi, E., Mazzetti, M., Ravina, A., Annunziato, F., De Carli, M., Piccinni, M. P., Manetti, R., Carbonari, M., Pesce, A. M., Del Prete, G. and *et al.* (1994). Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science* **265**, 244–248.

Maizels, R. M. (2009). Exploring the immunology of parasitism – from surface antigens to the hygiene hypothesis. *Parasitology* **136**, 1549–1564.

Maizels, R. M., Bundy, D. A. P., Selkirk, M. E., Smith, D. F. and Anderson, R. M. (1993). Immunological modulation and evasion by helminth parasites in human populations. *Nature, London* **365**, 797–805.

Maizels, R. M., Gomez-Escobar, N., Gregory, W. F., Murray, J. and Zang, X. X. (2001). Immune evasion genes from filarial nematodes. *International Journal for Parasitology* **31**, 889–898.

Maizels, R. M. and Kurniawan-Atmadja, A. (2002). Variation and polymorphism in helminth parasites. *Parasitology* **125** (Suppl.) S25–S37.

Maizels, R. M., Pearce, E. J., Artis, D., Yazdanbakhsh, M. and Wynn, T. A. (2009). Regulation of pathogenesis and immunity in helminth infections. *Journal of Experimental Medicine* **206**, 2059–2066.

Maizels, R. M. and Yazdanbakhsh, M. (2003). Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature Reviews Immunology* **3**, 733–744.

Malhotra, I., Mungai, P., Wamachi, A., Kioko, J., Ouma, J. H., Kazura, J. W. and King, C. L. (1999). Helminth- and *Bacillus* Calmette-Guerin-induced immunity in children sensitized *in utero* to filariasis and schistosomiasis. *Journal of Immunology* **162**, 6843–6848.

Malhotra, I., Ouma, J., Wamachi, A., Kioko, J., Mungai, P., Omollo, A., Elson, L., Koech, D., Kazura, J. W. and King, C. L. (1997). *In utero* exposure to helminth and mycobacterial antigens generates cytokine responses similar to that observed in adults. *Journal of Clinical Investigation* **99**, 1759–1766.

Mayer, L. (2000). Mucosal immunity and gastrointestinal antigen processing. *Journal of Pediatric Gastroenterology and Nutrition* **30** (Suppl.), S4–S12.

Meamar, A. R., Rezaian, M., Mohraz, M., Hadighi, R. and Kia, E. B. (2007). *Strongyloides stercoralis* hyper-infection syndrome in HIV+/AIDS patients in Iran. *Parasitology Research* **101**, 663–665.

Medeiros, M., Figueiredo, J. P., Almeida, M. C., Matos, M. A., Araujo, M. I., Cruz, A. A., Atta, A. M., Rego, M. A. V., De Jesus, A. R., Taketomi, E. A. and Carvalho, E. M. (2003). *Schistosoma mansoni* infection is associated with a reduced course of asthma. *Journal of Allergy and Clinical Immunology* **111**, 947–951.

Mestas, J. and Hughes, C. C. W. (2004). Of mice and not men: differences between mouse and human immunology. *Journal of Immunology* **172**, 2731–2738.

Metenou, S., Dembele, B., Konate, S., Dolo, H., Coulibaly, S. Y., Coulibaly, Y. I., Diallo, A. A., Soumaoro, L., Coulibaly, M. E., Sanogo, D., Doumbia, S. S., Wagner, M., Traore, S. F., Klion, A., Mahanty, S. and Nutman, T. B. (2009). Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population. *Journal of Immunology* **183**, 916–924.

Montenegro, S. N. M., Miranda, P., Mahanty, S., Abath, F. G. C., Teixeira, K. M., Coutinho, E. M., Brinkman, J., Goncalves, I., Domingues, L. A. W., Domingues, A. A. C., Sher, A. and Wynn, T. A. (1999). Cytokine production in acute versus chronic human schistosomiasis mansoni: the cross-regulatory role of interferon-gamma and interleukin 10 in the responses of peripheral blood mononuclear cells and splenocytes to parasite antigens. *The Journal of Infectious Diseases* **179**, 1502–1514. doi: 10.1086/314748.

Moreno, Y. and Geary, T. G. (2008). Stage- and gender-specific proteomic analysis of *Brugia malayi* excretory-secretory products. *PLoS Neglected Tropical Diseases* **2**, e326.

Mountford, A. P. and Pearlman, E. (1998). Interleukin-12 and the host response to parasitic helminths; the paradoxical effect on protective immunity and immunopathology. *Parasite Immunology* **20**, 509–517.

Muok, E. M. O., Mwinzi, P. N. M., Black, C. L., Carter, J. M., Ng'ang'a, Z. W., Gicheru, M. M., Secor, W. E., Karanja, D. M. S. and Colley, D. G. (2009). Short report: childhood coinfections with *Plasmodium falciparum* and *Schistosoma mansoni* result in lower percentages of activated T cells and T regulatory memory cells than schistosomiasis only. *American Journal of Tropical Medicine and Hygiene* **81**, 370–370.

Mutapi, F., Burchmore, R., Mduluza, T., Foucher, A., Harcus, Y., Nicoll, G., Midzi, N., Turner, C. M. and Maizels, R. M. (2005). Praziquantel treatment of individuals exposed to *Schistosoma haematobium* enhances serological recognition of defined parasite antigens. *Journal of Infectious Diseases* **192**, 1108–1118.

Mutapi, F., Burchmore, R., Mduluza, T., Midzi, N., Turner, C. M. R. and Maizels, R. M. (2008). Age-related and infection intensity-related shifts in antibody recognition of defined protein antigens in a schistosome-exposed population. *Journal of Infectious Diseases* **198**, 167–175.

Mutapi, F., Hagan, P., Woolhouse, M. E. J., Mduluza, T. and Ndhlovu, P. D. (2003). Chemotherapy-induced, age-related changes in antischistosome antibody responses. *Parasite Immunology* **25**, 87–97.

Mutapi, F., Ndhlovu, P. D., Hagan, P. and Woolhouse, M. E. (2000). Anti-schistosome antibody responses in children coinfected with malaria. *Parasite Immunology* **22**, 207–209.

Mutapi, F., Ndhlovu, P. D., Hagan, P. and Woolhouse, M. E. J. (1997). A comparison of humoral responses to *Schistosoma haematobium* in areas with low and high levels of infection. *Parasite Immunology* **19**, 255–263.

Mutapi, F., Winborn, G., Midzi, N., Taylor, M., Mduluza, T. and Maizels, R. M. (2007). Cytokine responses to *Schistosoma haematobium* in a Zimbabwean population: contrasting profiles for IFN-gamma, IL-4, IL-5 and IL-10 with age. *BMC Infectious Diseases* **7**, 139.

Mwinzi, P. N. M., Karanja, D. M. S., Colley, D. G., Orago, A. S. S. and Secor, W. E. (2001). Cellular immune responses of schistosomiasis patients are altered by human immunodeficiency virus type 1 coinfection. *Journal of Infectious Diseases* **184**, 488–496.

Naus, C. W. A., Kimani, G., Ouma, J. H., Fulford, A. J. C., Webster, M., Van Dam, G. J., Deelder, A. M., Butterworth, A. E. and Dunne, D. W. (1999). Development of antibody isotype responses to *Schistosoma mansoni* in an immunologically naive immigrant population: Influence of infection duration, infection intensity, and host age. *Infection and Immunity* **67**, 3444–3451.

Novato-Silva, E., Gazzinelli, G. and Colley, D. G. (1992). Immune responses during human schistosomiasis mansoni. XVIII. Immunologic status of pregnant women and their neonates. *Scandinavian Journal of Immunology* **35**, 429–437.

Nutman, T. B. and Kumaraswami, V. (2001). Regulation of the immune response in lymphatic filariasis: perspectives on acute and chronic infection with *Wuchereria bancrofti* in South India. *Parasite Immunology* **23**, 389–399.

Opara, K. N., Udoidung, N. I. and Ukpong, I. G. (2007). Genitourinary schistosomiasis among pre-primary schoolchildren in a rural community within the Cross River Basin, Nigeria. *Journal of Helminthology* **81**, 393–397.

Osada, Y. and Kanazawa, T. (2010). Parasitic helminths: New weapons against immunological disorders. *Journal of Biomedicine and Biotechnology* **2010**, 743–758.

Parkinson, J., Mitreva, M., Whitton, C., Thomson, M., Daub, J., Martin, J., Schmid, R., Hall, N., Barrell, B., Waterston, R. H., McCarter, J. P. and Blaxter, M. L. (2004). A transcriptomic analysis of the phylum Nematoda. *Nature Genetics* **36**, 1259–1267.

Pastrana, D. V., Raghavan, N., FitzGerald, P., Eisinger, S. W., Metz, C., Bucala, R., Schleimer, R. P., Bickel, C. and Scott, A. L. (1998). Filarial nematode parasites secrete a homologue of the human cytokine macrophage migration inhibitory factor. *Infection and Immunity* **66**, 5955–5963.

Pearce, E. J. and MacDonald, A. S. (2002). The immunobiology of schistosomiasis. *Nature Reviews Immunology* **2**, 499–511.

Peisong, G., Mao, X. Q., Enomoto, T., Feng, Z., Gloria-Bottini, F., Bottini, E., Shirakawa, T., Sun, D. and Hopkin, J. M. (2004). An asthma-associated genetic variant of STAT6 predicts low burden of ascaris worm infestation. *Genes and Immunity* **5**, 58–62.

Pit, D. S., Polderman, A. M., Schulz-Key, H. and Soboslay, P. T. (2000). Prenatal immune priming with helminth infections: parasite-specific cellular reactivity and Th1 and Th2 cytokine responses in neonates. *Allergy* **55**, 732–739.

Pullan, R. L., Bethony, J. M., Geiger, S. M., Cundill, B., Correa-Oliveira, R., Quinnell, R. J. and Brooker, S. (2008). Human helminth co-infection: analysis of spatial patterns and risk factors in a brazilian community. *PLoS Neglected Tropical Diseases* **2**, e352.

Quinnell, R. J. (2003). Genetics of susceptibility to human helminth infection. *International Journal for Parasitology* **33**, 1219–1231.

Quinnell, R. J., Pritchard, D. I., Raiko, A., Brown, A. P. and Shaw, M. A. (2004). Immune responses in human necatoriasis: association between interleukin-5 responses and resistance to reinfection. *Journal of Infectious Diseases* **190**, 430–438.

Raso, G., Luginbuhl, A., Adjoua, C. A., Tian-Bi, N. T., Silue, K. D., Matthys, B., Vounatsou, P., Wang, Y., Dumas, M. E., Holmes, E., Singer, B. H., Tanner, M., N'Goran, E. K. and Utzinger, J. (2004). Multiple parasite infections and their relationship to self-reported morbidity in a community of rural Cote d'Ivoire. *International Journal of Epidemiology* **33**, 1092–1102.

Reddy, A. and Fried, B. (2007). The use of *Trichuris suis* and other helminth therapies to treat Crohn's disease. *Parasitology Research* **100**, 921–927.

Redman, E., Grillo, V., Saunders, G., Packard, E., Jackson, F., Berriman, M. and Gilleard, J. S. (2008). Genetics of mating and sex determination in the parasitic nematode *Haemonchus contortus*. *Genetics* **180**, 1877–1887.

Rudge, J. W., Stothard, J. R., Basanez, M. G., Mgeni, A. F., Khamis, I. S., Khamis, A. N. and Rollinson, D. (2008). Micro-epidemiology of urinary schistosomiasis in Zanzibar: Local risk factors associated with distribution of infections among schoolchildren and relevance for control. *Acta Tropica* **105**, 45–54.

Sartono, E., Kruize, Y. C. M., Kurniawan, A., Maizels, R. M. and Yazdanbakhsh, M. (1997). Depression of antigen-specific interleukin-5 and interferon-gamma responses in human lymphatic filariasis as a function of clinical status and age. *Journal of Infectious Diseases* **175**, 1276–1280.

Satti, M. Z., Lind, P., Vennervald, B. J., Sulaiman, S. M., Daffalla, A. A. and Ghalib, H. W. (1996). Specific immunoglobulin measurements related to exposure and resistance to *Schistosoma mansoni* infection in Sudanese canal cleaners. *Clinical and Experimental Immunology* **106**, 45–54.

Schramm, G., Falcone, F. H., Gronow, A., Haisch, K., Mamat, U., Doenhoff, M. J., Oliveira, G., Galle, J., Dahinden, C. A. and Haas, H. (2003). Molecular characterization of an interleukin-4-inducing factor from *Schistosoma mansoni* eggs. *Journal of Biological Chemistry* **278**, 18384–18392.

Scott, J. T., Turner, C. M. R., Mutapi, F., Woolhouse, M. E. J., Chandiwana, S. K., Mduluza, T., Ndhlovu, P. D. and Hagan, P. (2000). Dissociation of interleukin-4 and interleukin-5 production following treatment for *Schistosoma haematobium* infection in humans. *Parasite Immunology* **22**, 341–348.

Scrivener, S., Yemaneberhan, H., Zebenigus, M., Tilahun, D., Girma, S., Ali, S., McElroy, P., Custovic, A., Woodcock, A., Pritchard, D., Venn, A. and Britton, J. (2001). Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study. *Lancet* **358**, 1493–1499.

Secor, W. E., Karanja, D. M. S. and Colley, D. G. (2004). Interactions between schistosomiasis and human immunodeficiency virus in western Kenya. *Memorias do Instituto Oswaldo Cruz* **99**, 93–95.

Semnani, R. T., Law, M., Kubofcik, J. and Nutman, T. B. (2004). Filaria-induced immune evasion: suppression by the infective stage of *Brugia malayi* at the earliest host-parasite interface. *Journal of Immunology* **172**, 6229–6238.

Semnani, R. T., Sabzevari, H., Iyer, R. and Nutman, T. B. (2001). Filarial antigens impair the function of human dendritic cells during differentiation. *Infection and Immunity* **69**, 5813–5822.

Sentongo, E., Rubaale, T., Buttner, D. W. and Brattig, N. W. (1998). T cell responses in coinfection with *Onchocerca volvulus* and the human immunodeficiency virus type 1. *Parasite Immunology* **20**, 431–439.

Silveira, A. M. S., Gazzinelli, G., Alves-Oliveira, L. F., Bethony, J., Gazzinelli, A., Carvalho-Queiroz, C., Alvarez, M. C. B., Lima-Silva, F. C., Prata, A., LoVerde, P. T. and Correa-Oliveira, R. (2004). Human *Schistosomiasis mansoni*: intensity of infection differentially affects the production of interleukin-10, interferon-gamma and interleukin-13 by soluble egg antigen or adult worm antigen stimulated cultures. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **98**, 514–519.

Smith, H. M., DeKaminsky, R. G., Niwas, S., Soto, R. J. and Jolly, P. E. (2001). Prevalence and intensity of infections of *Ascaris lumbricoides* and *Trichuris trichiura* and associated socio-demographic variables in four rural Honduran communities. *Memorias do Instituto Oswaldo Cruz* **96**, 303–314.

Smithers, S. R. and Terry, R. J. (1967). Resistance to experimental infection with *Schistosoma mansoni* in rhesus monkeys induced by the transfer of adult worms. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **61**, 517–533.

Sousa-Figueiredo, J. C., Basanez, M. G., Mgeni, A. F., Khamis, I. S., Rollinson, D. and Stothard, J. R. (2008). A parasitological survey, in rural Zanzibar, of pre-school children and their mothers for urinary schistosomiasis, soil-transmitted helminthiases and malaria, with observations on the prevalence of anaemia. *Annals of Tropical Medicine and Parasitology* **102**, 679–692.

Steel, C., Guinea, A., McCarthy, J. S. and Ottesen, E. A. (1994). Long-term effect of prenatal exposure to maternal microfilaraemia on immune responsiveness to filarial parasite antigens. *Lancet* **343**, 890–893.

Steel, C. and Nutman, T. B. (2003). CTLA-4 in filarial infections: implications for a role in diminished T cell reactivity. *Journal of Immunology* **170**, 1930–1938.

Tchuem Tchuente, L. A., Behnke, J. M., Gilbert, F. S., Southgate, V. R. and Vercruysse, J. (2003). Polyparasitism with *Schistosoma haematobium* and soil-transmitted helminth infections among school children in Loum, Cameroon. *Tropical Medicine & International Health* **8**, 975–986.

Tingley, G. A., Butterworth, A. E., Anderson, R. M., Kariuki, H. C., Koech, D., Mugambi, M., Ouma, J. H., Siongok, T. K. A. and Sturrock, R. F. (1988). Predisposition of humans to infection with *Schistosoma mansoni* – evidence from the reinfection of individuals following chemotherapy. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **82**, 448–452.

Trottein, F., Godin, C., Pierce, R. J., Sellin, B., Taylor, M. G., Gorillot, I., Silva, M. S., Lecocq, J. P. and Capron, A. (1992). Interspecies variation of

schistosome 28-Kda glutathione-S-transferases. *Molecular and Biochemical Parasitology* **54**, 63–72.

Trpis, M. (2006). Consequences of vector behaviour in epidemiology of onchocerciasis on the Firestone Rubber Plantation in Liberia. *American Journal of Tropical Medicine and Hygiene* **74**, 833–840.

Turaga, P. S. D., Tierney, T. J., Bennett, K. E., McCarthy, M. C., Simonek, S. C., Enyong, P. A., Moukatte, D. W. and Lustigman, S. (2000). Immunity to onchocerciasis: cells from putatively immune individuals produce enhanced levels of interleukin-5, gamma interferon, and granulocyte-macrophage colony-stimulating factor in response to *Onchocerca volvulus* larval and male worm antigens. *Infection and Immunity* **68**, 1905–1911.

Turner, J. D., Faulkner, H., Kamgno, J., Cormont, F., Van Snick, J., Else, K. J., Grencis, R. K., Behnke, J. M., Boussinesq, M. and Bradley, J. E. (2003). Th2 cytokines are associated with reduced worm burdens in a human intestinal helminth infection. *Journal of Infectious Diseases* **188**, 1768–1775.

Veldhoen, M., Uyttenhove, C., Van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C. and Stockinger, B. (2008). Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature Immunology* **9**, 1341–1346.

Vermund, S. H., Bradley, D. J. and Ruiztiben, E. (1983). Survival of *Schistosoma mansoni* in the human host – estimates from a community-based prospective-study in Puerto-Rico. *American Journal of Tropical Medicine and Hygiene* **32**, 1040–1048.

Viana, I. R., Sher, A., Carvalho, O. S., Massara, C. L., Eloi-Santos, S. M., Pearce, E. J., Colley, D. G., Gazzinelli, G. and Correa-Oliveira, R. (1994). Interferon-gamma production by peripheral blood mononuclear cells from residents of an area endemic for *Schistosoma mansoni*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**, 466–470.

Viana, I. R. C., CorreaOliveira, R., Dossantos, O., Massara, C. L., Colosimo, E., Colley, D. G. and Gazzinelli, G. (1995). Comparison of antibody isotype responses to *Schistosoma mansoni* antigens by infected and putative resistant individuals living in an endemic area. *Parasite Immunology* **17**, 297–304.

Watanabe, K., Mwinzi, P. N., Black, C. L., Muok, E. M., Karanja, D. M., Secor, W. E. and Colley, D. G. (2007). T regulatory cell levels decrease in people infected with *Schistosoma mansoni* on effective treatment. *American Journal of Tropical Medicine and Hygiene* **77**, 676–682.

Webster, M., CorreaOliveira, R., Gazzinelli, G., Viana, I. R. C., Fraga, L. A. D., Silveira, M. S. and Dunne, D. W. (1997). Factors affecting high and low human IgE responses to schistosome worm antigens in an area of Brazil endemic for *Schistosoma mansoni* and hookworm. *American Journal of Tropical Medicine and Hygiene* **57**, 487–494.

Williams-Blangero, S., McGarvey, S. T., Subedi, J., Wiest, P. M., Upadhayay, R. P., Rai, D. R., Jha, B., Olds, G. R., Wu, G. L. and Blangero, J. (2002*a*). Genetic component to susceptibility to *Trichuris trichiura*: Evidence from two Asian populations. *Genetic Epidemiology* **22**, 254–264.

Williams-Blangero, S., VandeBerg, J. L., Subedi, J., Aivaliotis, M. J., Rai, D. R., Upadhayay, R. P., Jha, B. and Blangero, J. (2002*b*). Genes on chromosomes 1 and 13 have significant effects on *Ascaris* infection. *Proceedings of the National Academy of Sciences, USA* **99**, 5533–5538.

Williams, M. E., Montenegro, S., Domingues, A. L., Wynn, T. A., Teixeira, K., Mahanty, S., Coutinho, A. and Sher, A. (1994). Leukocytes of patients with *Schistosoma mansoni* respond with a Th2 pattern of cytokine production to mitogen or egg antigens but with a Th0 pattern to worm antigens. *Journal of Infectious Diseases* **170**, 946–954.

Wilson, S., Jones, F. M., Mwatha, J. K., Kimani, G., Booth, M., Kariuki, H. C., Vennervald, B. J., Ouma, J. H., Muchiri, E. and Dunne, D. W. (2008). Hepatosplenomegaly is associated with low regulatory and Th2 responses to schistosome antigens in childhood schistosomiasis and malaria coinfection. *Infection and Immunity* **76**, 2212–2218.

Woolhouse, M. E. J. (1998). Patterns in parasite epidemiology: the peak shift. *Parasitology Today* **14**, 428–434.

Woolhouse, M. E. J. and Hagan, P. (1999). Seeking the ghost of worms past. *Nature Medicine* **5**, 1225–1227.

Wynn, T. A., Oswald, I. P., Eltoum, I. A., Caspar, P., Lowenstein, C. J., Lewis, F. A., James, S. L. and Sher, A. (1994). Elevated expression of Th1 cytokines and nitric-oxide synthase in the lungs of vaccinated mice after challenge infection with *Schistosoma mansoni*. *Journal of Immunology* **153**, 5200–5209.

Yazdanbakhsh, M. and Sacks, D. L. (2010). Why does immunity to parasites take so long to develop? *Nature Reviews Immunology* **10**, 80–81.

Zhang, W. B., Ross, A. G. and McManus, D. P. (2008). Mechanisms of immunity in hydatid disease: Implications for vaccine development. *Journal of Immunology* **181**, 6679–6685.

Zhou, Y., Zheng, H. J., Chen, Y. Y., Zhang, L., Wang, K., Guo, J., Huang, Z., Zhang, B., Huang, W., Jin, K., Dou, T. H., Hasegawa, M., Wang, L., Zhang, Y., Zhou, J., Tao, L., Cao, Z. W., Li, Y. X., Vinar, T., Brejova, B., Brown, D., Li, M., Miller, D. J., Blair, D., Zhong, Y., Chen, Z., Hu, W., Wang, Z. Q., Zhang, Q. H., Song, H. D., Chen, S. J., Xu, X. N., Xu, B., Ju, C., Huang, Y. C., Brindley, P. J., McManus, D. P., Feng, Z., Han, Z. G., Lu, G., Ren, S. X., Wang, Y. Z., Gu, W. Y., Kang, H., Chen, J., Chen, X. Y., Chen, S. T., Wang, L. J., Yan, J., Wang, B. Y., Lv, X. Y., Jin, L., Wang, B. F., Pu, S. Y., Zhang, X. L., Zhang, W., Hu, Q. P., Zhu, G. F., Wang, J., Yu, J., Wang, J., Yang, H. M., Ning, Z. M., Beriman, M., Wei, C. L., Ruan, Y. J., Zhao, G. P., Wang, S. Y., Liu, F., Wang, Z. Q., Zheng, H. J., Zhang, Q. H., Wang, S. Y., Han, Z. G. and Seque, S. J. G. (2009). The *Schistosoma japonicum* genome reveals features of host-parasite interplay. *Nature, London* **460**, 345–U356.