Innate, adaptive and regulatory immune responses in human schistosomiasis in Gabon

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1

General introduction.

1

Background

More than 1.5 billion people, or 24% of the world's population are infected with parasitic helminth infections worldwide [1]. A parasitic relationship is one in which one species, the parasite, lives off and benefits at the expense of the other, the host. Helminths have lived in what until recently has been considered a non-mutual symbiotic relationship with humans for millennia. The earliest documented case of human schistosomiasis occurred over 5000 years ago [2] and of concurrent infection with *Schistosoma haematobium, Taenia species, Trichinella spiralis*, and *Plasmodium falciparum* at approximately 3000 years ago [3]. The co-existence and co-evolution of worms and humans has profoundly shaped and altered the human immune response and even the human genetic composition. Greater diversity and load of parasitic worms has been correlated with more extensive single nucleotide polymorphisms (SNP) variation in immunological loci and reduced incidence of immunopathological diseases which have been linked to genetic loci that increase the likelihood of developing these diseases in Western countries [4]. Considering this co-evolution, responses which may be beneficial under strong infection pressure may now result in the development of undesirable pathologies.

Until recently, the epidemiological course of human diseases had substantially remained unchanged with a life expectancy of 40 or 50 years [5]. However during the last decades of the 19th century and throughout the 20th century, improvements in healthcare and hygiene have revolutionized the epidemiological landscape in Westernized countries, with a significant increase in human lifespan and a shift from infectious to chronic degenerative diseases as prevailing causes of death [6]. A larger incidence of allergy, autoimmune disorders, cardiovascular diseases and metabolic disorders has been observed; interestingly all of these diseases have a strong chronic inflammatory component. Although in low- to middle-income countries the prevalence of helminths is still high in rural areas, urban centers have seen a significant decrease in helminth infections accompanied by an increase in non-communicable disorders [7].

It has been proposed that the decrease in infectious diseases is directly related to the increase in hyper-inflammatory disorders due to insufficient education of the immune system by microbes and parasites and an insufficient development and maturation of the regulatory arm of the immune response leading to uncontrolled inflammatory responses against innocuous or self-antigens [8]. Numerous epidemiological studies show that in populations with high rates of parasitic infections the prevalence of allergic diseases, such as asthma, eczema and allergic rhinitis, is significantly lower [9]. The protective effect of helminth infections is very likely due to the down-regulation of the immune system by the parasite during chronic infection to ensure its own survival, which extends to bystander allergens and leads to defects in the establishment of immune tolerance.

The mechanisms and consequences of immunomodulation induced by helminth infections are therefore of tremendous interest as it now seems that the relationship between helminths and humans may in fact in some contexts be mutually symbiotic, although this is a rather fine balance and the negative consequences of parasitic infection may still result in excessive morbidity if the level and severity of infection is not controlled. Indeed helminths and more ideally helminth derived molecules with immunomodulatory capacities are currently

investigated as targets for therapeutic applications in the treatment or even prevention of hyper-inflammatory disorders [10]. On the other hand the down-regulation of the immune response induced by helminth infections may adversely affect immune responses against other pathogens, the efficacy of drug treatments and may furthermore present challenges for the delivery of prophylactic vaccines where a hyper-responsive immune system is desirable.

Consequently an investigative approach which studies the immune response in all of its complexity simultaneously should provide novel insights which can then be harnessed and exploited to either abrogate or conversely strengthen suppressed immune responses in order to respectively combat helminth infections and inflammatory diseases.

This thesis is centered around schistosomiasis and the way the immune system is affected during this infection. In the following two sections, the parasite life cycle will be introduced and important aspects of immune responses to these parasites will be described.

Schistosomiasis

Schistosomiasis, also known as bilharzia, is a chronic parasitic disease caused by trematodes belonging to the genus *Schistosoma*. Several species of schistosomes can infect humans, of which *S. haematobium*, *S. mansoni* and *S. japonicum* are most prevalent, while *S. intercalatum*, *S. mekongi* and *S. guineensis* have a more limited distribution. The disease is found in tropical and sub-tropical areas of Africa, Asia, South America, the Caribbean and in the Middle East. Approximately 85% of the world's cases of schistosomiasis are in Africa, where prevalence rates can exceed 50% in local populations and infections are most prevalent in poor communities without access to safe drinking water and adequate sanitation (the schistosome life cycle is illustrated in Figure 1). Schistosomiasis affects almost 240 million people worldwide, and more than 700 million people live in endemic areas. It is estimated that more than 200,000 deaths per year are due to schistosomiasis in sub-Saharan Africa and several million are considered to suffer from severe consequences of the infection. Schistosome infections rank second only to malaria in terms of morbidity, imposing considerable burden on the social and economic development of communities in endemic countries. Schistosomiasis is readily treated with praziquantel; however, as treatment is not preventative, a large portion of the population in endemic areas requires regular treatment due to continuous re-exposure and a high rate of re-infection. It is for this reason that a vaccine against schistosomiasis is needed and remains the most potentially effective means for the control of this disease [11].

A short introduction to immune responses during schistosome infection

Epidemiological studies among populations in which schistosomiasis is endemic provide evidence that age-dependent partial immunity against schistosomes can develop with age and that resistance to schistosomiasis is gradually acquired and can be attributed to cumulative exposure to infection. Protective IgA, IgE and IgG levels have been demonstrated [12,13], and resistance to (re)-infection is correlated with an increased ratio of IgE (low in children, high in adults) and IgG4 (high in children, low in adults) [14] and increased levels of CD23 (the low-affinity IgE receptor (FcγRII))$^{+}$ B cells [15,16]. Antibody responses clearly play an important role in the control of schistosome infections, yet surprisingly little is known about the phenotype

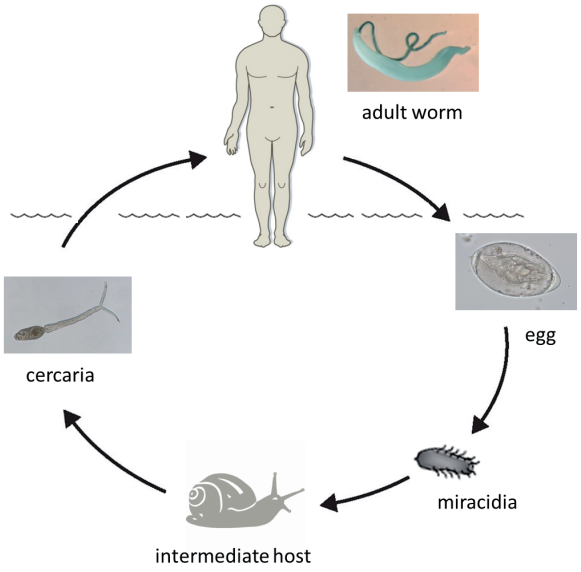

Figure 1: Life cycle of schistosomes
Snails are the intermediate host and humans the definitive hosts in the schistosome transmission cycle. Following penetration of the human skin the cercariae transform into schistosomula, which migrate in the blood stream through the lungs to the liver, where the female and male worms mature and develop into schistosomes. After mating the adult worms migrate in the case of intestinal schistosomiasis to the mesenteric vessels lining the intestine, or in the case of urinary schistosomiasis to the vessels of the bladder. The female worm lays eggs, some of which get trapped in the surrounding tissue and the rest are excreted with stool or urine. When the eggs come in contact with fresh water, they mature into miracidia which penetrate the snail host where they undergo asexual changes and develop into cercariae [11]. Photographs courtesy of E.A.T. Brienen.

and function of B cells in the course of schistosomiasis. Recent technical developments, such as immunoglobulin analysis with flow cytometry, have allowed us to study B cell biology in much greater detail.

Whole blood assays on the other hand provide a simple technological solution, while keeping the cells in their natural environment and *in vivo* composition, to study in larger numbers of individuals the innate and adaptive cytokine responses [17]. Innate immune responses are not well characterized in the context of human schistosomiasis. Toll-like receptors (TLRs), the most extensively studied class of pathogen recognition receptors (PRRs), have been shown to be altered in *S. haematobium*-infected individuals [18,19]. Moreover, another class of PRRs, the C-type lectins (CLRs), have also been shown to be important for recognition of schistosomal ligands [20]. However, relatively little is known about innate immune responses in human schistosomiasis in particular in terms of longitudinal studies. A greater understanding of the innate immune response and of the molecules that regulate and activate it may provide a way to manipulate the immune system such that beneficial responses will be enhanced and deleterious ones ameliorated.

Adaptive responses during the course of schistosome infection are relatively well

characterized. Acute stages of schistosome infection are characterized by a dominant T helper 1 (Th1)-mediated immune response, hallmarked by high levels of interferon (IFN)-γ. This changes markedly following parasite maturation and egg production; Th1 responses are down-regulated and a strong Th2 response emerges typified by high production of IL-4, IL-5, IL-13, IgE synthesis, and eosinophilia [21]. Maintaining a balanced and controlled Th1 and Th2 response ensures formation of protective granulomas around parasite eggs without excessive pathology thereby minimizing host immunopathology.

Hypo-responsiveness is another feature of chronic infection which results in reduced proliferation and cytokine production which are thought to contribute not only to keeping pathology at bay but also allow parasite survival. The effect of long-term anthelminthic treatment on adaptive immune responses is still not fully understood, nor is the relationship between adaptive and regulatory responses in the course of infection. While Th2 responses are instrumental in modulating potentially life-threatening outcomes in the initial stages of infection, excessive and prolonged Th2 responses contribute to the development of pathology and chronic morbidity. During the chronic phase of infection, Th2 responses are in turn modulated by regulatory responses, leading to diminished Th2 responses and a reduction in granuloma size. Regulatory responses in schistosomiasis have been predominantly attributed to increased levels of Forkhead box protein 3 $(FOXP3)^+$ regulatory T cells (Treg), which express molecules involved in the inhibition of immune responses, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and secrete the suppressory cytokines, IL-10 and TGF-β; together these lead to down-modulation of effector responses [22–24]. More recently IL-10 producing regulatory B cells (Breg) have been shown to be associated with helminth infections and modulation of inflammatory responses [25]. Regulatory B cells are now known to influence T cell proliferation, down-regulate $CD4^+$, $CD8^+$, NK T cell activation and promote $FOXP3^+$ Treg induction [26].

Regulatory responses induce immune hypo-responsiveness characterized by reduced *in vitro* proliferation of T lymphocytes and decreased Th1- and Th2-type cytokine production not only to schistosomal antigens but also to third party antigens including allergens, vaccines or self-antigens [27–31]. Indeed the relationship between helminth infections and reduced prevalence and severity of not only auto-immune disease but also metabolic and cardiovascular diseases is currently an area of intense investigation. Central to studies that aim to understand how this infection should be combatted or how this infection might relate to inflammatory diseases is an in depth characterization of the immune response.

Outline and aims of this thesis

The general objective of this thesis is to study the characteristics of the immune response during schistosomiasis, caused by chronic infection, in an endemic setting. An innovative approach of concurrently investigating innate, adaptive and regulatory responses will further increase our fundamental knowledge of how these responses are connected during helminth infection. While field studies in endemic settings may be logistically challenging, there is no substitute for real-life biological settings of infection. Moreover, these studies are of great importance for bilateral knowledge transfer and establishing collaborations with scientists in low-resource

settings.

More specifically several aims can be distinguished. Innate immune responses have not been studied in much detail previously and may therefore provide novel targets for therapy with a view to enhancing beneficial responses or ameliorating deleterious ones. To this end we investigate in detail whole blood innate responses not only between *S. haematobium*-infected and uninfected schoolchildren but also between African and European populations. Regulatory responses are key to parasite survival and reduced host tissue damage and therefore further understanding of regulatory networks and mechanisms may contribute to new possibilities for control of infection and pathology. Thus, we investigate both regulatory T cells and the more recently described regulatory B cells in our populations. Furthermore we characterize B cells in terms of antibody isotypes and different memory subsets during infection. Adaptive antigen specific responses are also measured to allow an integrated characterization of the various arms of the immune response during *S. haematobium* infection and how they relate to each other.

Study population and study design

The studies described in this thesis were conducted between April 2008 and September 2009 at Centre de Recherches Médicales de Lambaréné (CERMEL; formerly Medical Research Unit (MRU) [32]) (Figure 2) of the Albert Schweitzer Hospital, Lambaréné, Gabon. The city counts 35,000 inhabitants, with another 50,000 living in the surrounding rural areas, and is located 75 km south of the equator in the Central African Rainforest. The hospital is situated at the banks of the Ogooué River – one of the largest rivers in Central Africa, in the Moyen- Ogooué province. Study participants were recruited from local schools either from the semi-urban Lambaréné or from the surrounding rural villages within an approximate 30 km radius. The majority of inhabitants in the urban area have access to tap water or public wells however in the rural areas streams neighbouring the houses represent the main source of drinking water. Latrines are common, even in the urbanised areas. Income is generated mainly from farming, fishing and services. Gabon has a rainy season from September to May, broken up by a short dry period from December to January, and a longer dry season from June to September. *Plasmodium falciparum* malaria, schistosomiasis as well as soil-transmitted helminths including hookworm, *Ascaris lumbricoides* and *Trichuris trichiura* are endemic in the area [33,34].

All study participants were screened for helminth and malaria infections prior to inclusion in the studies. Haemogram data was likewise collected. For all the studies heparinized blood was collected from participants and processed according to study protocols at the CERMEL laboratory. All of the studies performed included schoolchildren infected with *S. haematobium* and comparable uninfected control schoolchildren. The first 2 studies included in this thesis had a cross-sectional design, whereas the later studies were longitudinal in nature. For the longitudinal studies the schoolchildren received praziquantel every 2 months between baseline and follow-up measurements 6-7 months later. For a subset of the individuals measurements were also taken at 6 weeks post-treatment. Field work was coordinated and carried out by local field staff.

For the comparison between African and European children, peripheral blood samples from Dutch children were also collected.

Figure 2: Study area and the CERMEL laboratory.

Approaches to characterization of immune responses during schistosome infection

In **Chapters 2**, **3** and **4** we utilize the whole blood assay to investigate both innate and adaptive immune responses in schoolchildren. This assay was developed to measure immune responses *ex vivo* and is ideal for field conditions due to small blood volume needed and no need for cell separation. For innate responses we targeted innate receptors including TLRs and CLRs and to measure adaptive responses we stimulated cells with schistosomal antigens. In **Chapters 4-7** peripheral blood mononuclear cells (PBMCs) isolated by Ficoll-Hypaque density gradient centrifugation were used for more detailed immunological studies. In **Chapters 5-7** magnetic-activated cell sorting (MACS) was used to separate B cells on the basis of surface CD19 antigen expression and to deplete regulatory T cells on the basis of CD25 expression from PBMCs. Isolated B cells were then cultured with different stimuli to understand the effect of infection on B cell biology. Total PBMCs or Treg depleted PBMCs were cultured with different antigens to delineate the role that Tregs play in controlling antigen specific responses. Enzyme Linked Immunosorbent Assay (ELISA) or Luminex were used to quantify cytokines and antibodies whereas flow cytometry (FACS) was used to characterize in detail various regulatory and memory B and T cell subsets in circulating PBMC populations and to measure proliferation rates in cultured PBMCs.

Scope and outline of this thesis

Innate and adaptive, as well as regulatory immune responses of individuals with and without *S. haematobium* infection were studied by developing and applying field applicable methods to the study population described above.

In **Chapter 2**, we compare PRR responses between African and European schoolchildren using identical reagents and experimental protocols in order to assess whether innate responses are affected by environmental factors.

In **Chapter 3** we assess cross-sectionally how innate and adaptive immune responses vary between *S. haematobium*-infected schoolchildren and uninfected controls.

In **Chapter 4** we compare immune responses of *S. haematobium*-infected children before and after treatment with praziquantel. Uninfected subjects were also followed up to control for any technical and seasonal effects on immunological parameters measured.

In **Chapter 5** we analyze regulatory T cell responses before and after praziquantel treatment, and we add to our phenotypic studies of these cells, the assessment of their functional activity by performing Treg depletion experiments.

Recently regulatory B cells have also been shown to be important players in immune regulation. In **Chapter 6** we investigate whether schistosome infection can induce functional Breg cells.

Antibody responses play a key role in the control of *S. haematobium* infections, yet the phenotype and function of B cells in human schistosomiasis has not been studied extensively. In **Chapter 7** we compare circulating memory B cell subsets in schistosome infected and uninfected schoolchildren and assess their response to B cell receptor (BCR) and TLR stimulation.

Finally, the main findings presented in this thesis are evaluated in a general discussion in **Chapter 8**.

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2

Differences in innate cytokine responses between European and African children.

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Abstract

Although differences in immunological responses between populations have been found in terms of vaccine efficacy, immune responses to infections and prevalence of chronic inflammatory diseases, the mechanisms responsible for these differences are not well understood. Therefore, innate cytokine responses mediated by various classes of pattern-recognition receptors including Toll-like receptors (TLR), C-type lectin receptors (CLRs) and nucleotide-binding oligomerisation domain-like receptors (NLRs) were compared between Dutch (European), semi-urban and rural Gabonese (African) children. Whole blood was stimulated for 24 hours and the pro-inflammatory tumor necrosis factor (TNF) and the anti-inflammatory/regulatory interleukin-10 (IL-10) cytokines in culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA). Gabonese children had a lower pro-inflammatory response to poly(I:C) (TLR3 ligand), but a higher pro-inflammatory response to FSL-1 (TLR2/6 ligand), Pam3 (TLR2/1 ligand) and LPS (TLR4 ligand) compared to Dutch children. Anti-inflammatory responses to Pam3 were also higher in Gabonese children. Non-TLR ligands did not induce substantial cytokine production on their own. Interaction between various TLR and non-TLR receptors was further assessed, but no differences were found between the three populations. In conclusion, using a field applicable assay, significant differences were observed in cytokine responses between European and African children to TLR ligands, but not to non-TLR ligands.

Introduction

Geographical variations in responses to vaccines have been reported; for example, protection against pulmonary tuberculosis by the Bacillus Calmette–Guérin (BCG) vaccine varies from 0% to 80% in adults, with higher protection in populations living at higher latitudes [1], or the rotavirus vaccine which provided little protection against rotavirus diarrhea in Gambian infants but showed promising results in Europe [2]. With respect to immunological changes following vaccination, a study in infants and adolescents from the United Kingdom showed that BCG vaccination induced a marked increase in interferon-γ (IFN-γ) response to Mycobacterium tuberculosis purified protein derivative (Mtb PPD), but not in infants and adolescents from Malawi [3]. Differences in the innate immune system and immune responsiveness caused by factors such as genetic variability and/or environmental exposures are thought in part to underlie the varying vaccine efficacies between populations.

Pattern-recognition receptors (PRRs) enable the innate immune system to recognize pathogens through interaction with pathogen-associated molecular patterns. Activation of the innate immune system via the PRRs induces cytokine production and expression of costimulatory molecules, which in turn control the activation of the adaptive immune system [4,5]. Several PRR families have been identified, including the Toll-like receptors (TLRs), the cytosolic nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and the cell-surface C-type lectin receptors (CLRs), each with their own specificities and signaling pathways. Integration of information from multiple PRRs enables the immune system to tailor its response, for example through cytokine production, to counteract specific exogenous and endogenous dangers [5,6]. As PRRs form the starting point of the innate immune response, which ultimately shapes the adaptive immune response, the magnitude as well as the type of cytokines (e.g. pro- or anti-inflammatory) produced in response to stimulation of these receptors could have a major impact on health. Alterations in PRR responses can determine responses not only to vaccines or pathogens but also to commensals, allergens or self-antigens [7–9].

Geographical differences in PRR responsiveness might also be linked to inflammatory-related diseases that are found to differ between populations. In high-income countries, allergic diseases like asthma, hay fever and eczema [10], and chronic inflammatory disorders such as type 1 diabetes mellitus [11,12], multiple sclerosis and inflammatory bowel disease [13] have increased, while the prevalence of these diseases is still low in low-income countries [14–16]. Although a better understanding of immunological differences could aid in the development of population-specific vaccines and treatment modalities against infectious, allergic and chronic inflammatory diseases, few studies have so far compared PRR responsiveness between populations.

The present study is part of the SCHISTOINIR project that aims to explore innate immune responses in schistosomiasis (www.york.ac.uk/res/schistoinir). The aim of this study was to compare PRR responsiveness between European, semi-urban African and rural African populations. To that end, pro- and anti-inflammatory responses to various TLR and non-TLR ligands were compared, using identical methods and reagents, between children living in the Netherlands, and those living in semi-urban and rural Gabon. Significant differences in TLR responsiveness in whole blood were found, with a reduced pro-inflammatory response to TLR3

stimulation and enhanced pro-inflammatory response to TLR2/1, TLR2/6 and TLR4 stimulation for Gabonese children as compared to Dutch children.

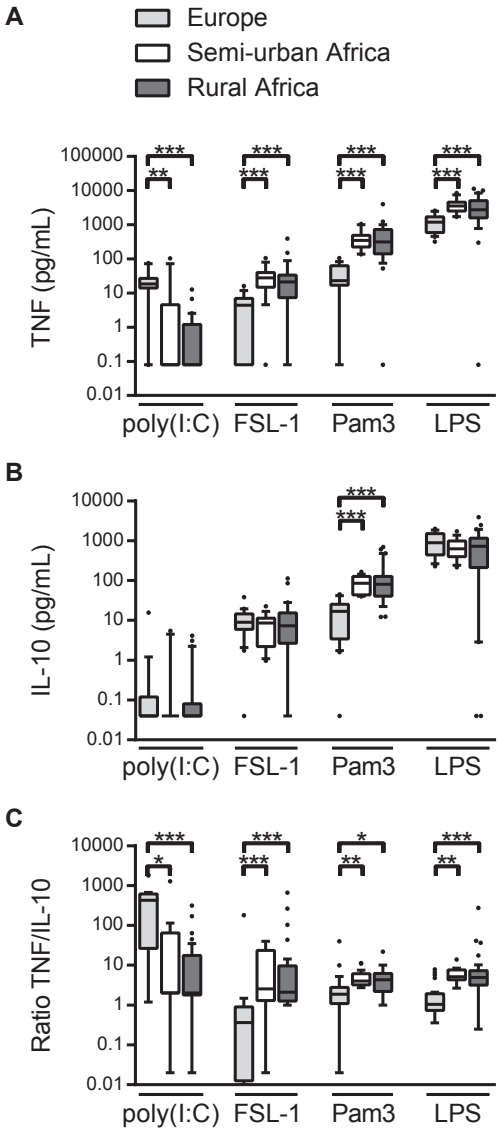

Figure 1. Whole blood cytokine responses to TLR ligands.
A) TNF responses to poly(I:C), FLS-1, Pam3 and LPS in European children (the Netherlands), and semi-urban and rural African children (Gabon). B) IL-10 responses to TLR stimulation. C) Pro/anti-inflammatory ratio as calculated by TNF/IL-10 ratio. * p<0.05, ** p<0.01, *** p<0.001

Results

Study population

The study population consisted of 21 Dutch children (median age: 9y, age range: 8-11, sex ratio: 12m/9f), 15 semi-urban Gabonese children (median age: 10y, age range: 6-15, sex ratio: 7m/8f) and 30 rural Gabonese children (median age: 10y, age range: 7-16, sex ratio: 17m/13f). The groups did not differ significantly in distributions of age and sex. Gabonese children differed in positivity for *S. haematobium*; semi-urban children were negative for schistosomiasis, whereas 57% of rural children were positive for this infection. Furthermore, 17% of semi-urban children were infected with geohelminths (*Trichuris trichiura*), while 44% of rural children were geohelminth positive (hookworm, *Ascaris lumbricoides* and/or *Trichuris trichiura*). A single case of malaria and of microfilaria and 5 cases of fungal skin infections were also detected amongst the rural Gabonese children, and 1 case of fungal skin infection amongst the semi-urban children. Rural children had higher levels of leukocyte (median: $6.50 \cdot 10^9$/L vs. $9.15 \cdot 10^9$/L, p=0.003) and eosinophil (median: $0.28 \cdot 10^9$/L vs. $1.31 \cdot 10^9$/L, p=0.001) counts compared to semi-urban children. While there was no parasitological data for Dutch children, exposure to helminths and malaria is unlikely. The Dutch hematological reference values are $4.5\text{-}13.5 \cdot 10^9$/L for leukocyte count and $<0.4 \cdot 10^9$/L for eosinophil count [23].

Cytokine responses to TLR ligands in European and African children

To compare innate immune responses of Dutch children with semi-urban and rural Gabonese children, whole blood was stimulated with different TLR and non-TLR ligands. Following poly(I:C) (TLR3 ligand) stimulation, TNF responses were lower in Gabonese children as compared to Dutch children, while IL-10 responses were low for all groups (Figure 1A and B). This resulted in a lower TNF/IL-10 ratio for Gabonese children (Figure 1C). Conversely, TNF responses to FSL-1 (TLR2/6 ligand), Pam3 (TLR2/1 ligand) and LPS (TLR4 ligand) were higher in Gabonese children (Figure 1A); IL-10 responses to FSL-1 and LPS did not differ between the groups and response to Pam3 was higher in Gabonese children (Figure 1B). When considered as ratio, Gabonese children exhibited a higher TNF/IL-10 ratio in response to these TLR ligands (Figure 1C). No differences were found between semi-urban and rural Gabonese children in whole blood responses to any of the TLR ligands tested. Comparison of responses by *Schistosoma* or geohelminth infection status did not result in significant differences (data not shown).

Spontaneous production of cytokines (response to medium) was low (Supplementary figure 1A). Despite the low levels, spontaneous IL-10 production was significantly higher for rural Gabonese children as compared to Dutch children, with a similar trend for higher spontaneous TNF production. No differences were observed between the groups in response to the positive control, the mitogen PHA (Supplementary figure 1B).

Interaction between TLR and non-TLR ligands in European children

Non-TLR ligands iE-DAP (a NOD1 ligand), mannan (ligand of mannose receptor (MR) and/or dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)), curdlan (a Dectin-1 ligand) or KOdiA-PC (ligand of the scavenger receptor CD36/SR-B2 [24])

alone did not induce substantial TNF or IL-10 production (Supplementary figure 2). However, they did influence TLR-mediated responses significantly. A synergistic effect was observed for the production of TNF, in particular in the interaction between TLR receptors and Dectin-1, while for IL-10 production both synergistic and inhibitory effects were observed (Table 1 and Supplementary figure 3).

Table 1. Degree of interaction between TLR and non-TLR ligands in Dutch children.

	TNF				IL-10			
	poly(I:C)	**FSL-1**	**Pam3**	**LPS**	**poly(I:C)**	**FSL-1**	**Pam3**	**LPS**
iE-DAP	++		+	+		+	+	+
mannan	+	+	+				+	+
KOdiA-PC					–			+
curdlan		++	++	++		–		–

The degree of interaction between two ligands was calculated for e.g. poly(I:C) and iE-DAP by: $(TNF^{poly(I:C)+iE\text{-}DAP} - TNF^{medium}) / ((TNF^{poly(I:C)} - TNF^{medium}) + (TNF^{iE\text{-}DAP} - TNF^{medium}))$. Values above 1 are regarded as synergy (+), and above 2 as strong synergy (++). Values below 1 are regarded as inhibition (–). Shown are statistically significant differences ($p<0.05$) according to the Wilcoxon signed rank test, when comparing the response to combined stimulation with the sum of separate stimulation (after background subtraction). See Supplementary figure 3 for details.

Comparison of PRR interaction profiles in European and African children

Interactions between TLRs and NOD1 (by iE-DAP) and MR/DC-SIGN (by mannan) were further compared between Dutch children and semi-urban and rural Gabonese children. Combination of Pam3 and iE-DAP or mannan resulted in significant synergistic effects for both TNF and IL-10 in all groups of children; other combinations were more variable (Figure 2, Supplementary figure 4). Of interest, while in Dutch children combined stimulation with poly(I:C) and iE-DAP or mannan led to significant synergy, Gabonese children exhibited a tendency towards inhibitory responses.

Discussion

This is the first study to systematically examine innate immune responses to several classes of PPRs in European and African children that originate from a semi-urban and a close-by rural area. African children from both a semi-urban and a rural area were included to take into account differences in environmental factors and genetic diversity between the African and European populations.

We report that Gabonese children had a reduced pro-inflammatory response to TLR3-ligand poly(I:C) and an enhanced pro-inflammatory response to TLR2/6-ligand FSL-1, TLR2/1-ligand Pam3 and TLR4-ligand LPS as compared to Dutch children. We further show that the concomitant engagement of TLRs with the receptors NOD1, CD36, MR/DC-SIGN or Dectin-1 can modify TLR responses, but no clear differences in the responses between Dutch and semi-urban and rural Gabonese children were seen, indicating that innate immune responses to the

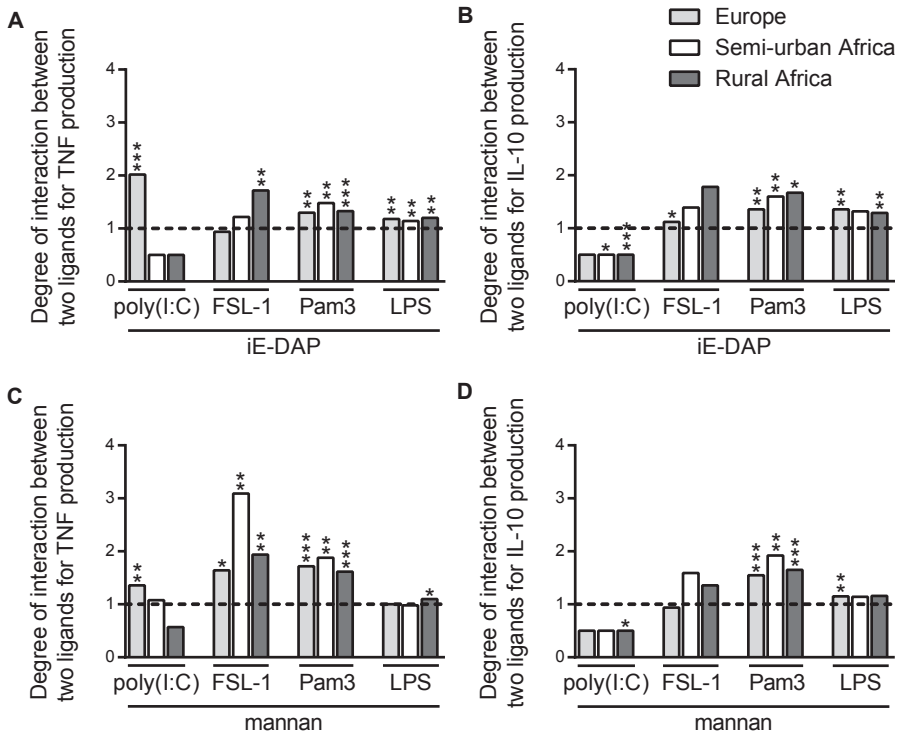

Figure 2. Degree of interaction between TLR and non-TLR ligands.
A) Degree of interaction for TNF production upon stimulation with poly(I:C), FSL-1, Pam3 or LPS combined with iE-DAP. This was calculated for e.g. poly(I:C) and iE-DAP by: ($TNF^{poly(I:C)+iE\text{-}DAP} - TNF^{medium}$) / (($TNF^{poly(I:C)} - TNF^{medium}$) + ($TNF^{iE\text{-}DAP} - TNF^{medium}$)). B) Degree of interaction for IL-10 production for combinations with iE-DAP. C) Degree of interaction for TNF production for combinations with mannan. D) Degree of interaction for IL-10 production for combinations with mannan.

non-TLR ligands studied are similar in European and Gabonese children.

Geographical differences in immune responses have not been studied extensively. One of the most field-applicable methods is the whole blood assay, which has been used here and in a few other studies to profile immune responses in populations residing in different geographic areas. In a recent study comparing responses of 2-year-old children to various PRRs across four continents, Smolen et al. [25], have shown that despite very distinct genetic and environmental background, the cytokine responses were largely similar in European, North American and South American children. However, the response of children from Africa was poor compared with the other three continents when stimulated with ligands to surface (TLR2, TLR4) or endosomal (TLR3, TLR7/8) PRRs but similar when targeting the cytosolic PRR (NOD2). However, our results are different. First, we find higher innate responses to LPS, Pam3 and FSL-1, stimuli which engage surface TLRs, in African (Gabonese) compared to European (Dutch) children. Currently, we are unable to explain the difference between our study and that of Smolen et al., but it has to be

emphasized that the age of the study subjects was substantially different in the two studies and differences in immune responses between Africans and Europeans has been found to be age-dependent [26]. Second, we see differences in immune responses between the genetically-distinct European and African children, but not in the genetically-similar semi-urban and rural African children. These results would suggest that genetic differences play an important role in the distinct innate responsiveness of African and European children. Polymorphisms have been described for TLR receptors and for molecules in downstream signaling pathways, for example for the gene coding for TIR domain-containing adapter protein/MyD88 adapter-like (TIRAP/Mal) that interacts with MyD88, and in TRIF [27]. Alternatively, it is possible that the difference between the semi-urban and rural African areas studied is too small to have a substantial effect on innate immune responsiveness.

The differences we observed in cytokine production in response to TLR ligands between Dutch and Gabonese children might be explained by variations in TLR expression levels. Van den Biggelaar et al. analyzed cord blood mononuclear cells (CBMCs) of newborns from a low-income country (Papua New Guinea; PNG) and a high-income country (Australia), showing less TNF and IL-10 in response to TLR2- and TLR4-specific stimulation, but more IFN-γ and IL-10 in response to BCG (recognized by TLR2, TLR4, and putatively TLR9) in PNG newborns [28]. The same study measured TLR mRNA expression and found that PNG CMBCs had higher TLR2 and TLR9 expression levels, but lower levels of TLR4. In contrast, Gabonese CBMCs have been shown to have lower TLR2 expression compared to Austrian CBMCs, but this was measured on freshly isolated cells that were fixed and then analyzed by flow cytometry [29] whereas the PNG study assessed mRNA expression in cryopreserved cells. Altogether, these data indicate how important technical differences can be when comparing studies conducted by different groups.

Downstream signaling pathways may also contribute towards differences observed in TLR responsiveness. TLR3 signals via Toll-IL-1 receptor (TIR) domain-containing adaptor inducing interferon-β (TRIF), while TLR2/6 and TLR2/1 signal via myeloid differentiation primary response protein 88 (MyD88) and TLR4 signals via both [30]. Thus, the enhanced pro-inflammatory response upon TLR3 stimulation in Dutch as compared to Gabonese children may be due to altered signaling via TRIF. Similarly, the enhanced pro-inflammatory response upon TLR2/6, TLR2/1 and TLR4 stimulation in Gabonese children may result from differences in MyD88 signaling. With the emergence of evidence for 'trained innate immunity' where a 'memory' of an earlier innate triggering in natural killer cells, macrophages and monocytes can be imprinted and can affect response to an innate ligand encountered later [31], it is possible that differential exposure to viruses, bacteria and parasites in the Dutch and Gabonese populations may explain the observed differences in innate responses [32–34].

Interaction of TLRs with other PRRs can modulate the type and the strength of an immune response. In Dutch children, combinations with curdlan resulted in the highest degree of synergy in TNF production, as described before [35]. Interestingly, despite the strong synergy between curdlan and TLRs that engage MyD88, no synergy was seen with the TLR3-ligand poly(I:C) which engages TIR. NOD1 has been found previously to synergize with TLR3 and TLR4 to induce IL-12p70 production [36], and our data indicate that synergy (by iE-DAP) can also

occur for TNF with TLR2/1, TLR3 and TLR4. Mannan was shown to synergize with TLR3, TLR2/6 and TLR2/1 for TNF production and with TLR2/6 and TLR4 for IL-10 production, in addition to the already described MR synergy with TLR2 [37]. CD36, stimulated by KOdiA-PC, did not synergize with TLRs for TNF production, while IL-10 production was variably affected.

The degree of interaction between PRRs did not differ considerably between Dutch children and those from semi-urban and rural Gabon. However, synergy between iE-DAP or mannan with poly(I:C) for TNF production occurred for Dutch children, but not for Gabonese children. This might suggest that NOD1 and MR are expressed to a lower extent in Gabonese children. However, when stimulating with LPS or FSL-1, children from rural Gabon showed higher activity of MR and NOD1 respectively. Therefore, it is unlikely that receptor expression explains the differences observed but rather the downstream pathways involved might be differently shaped.

Innate responses did not differ significantly between semi-urban and rural Gabonese children, and furthermore *S. haematobium* infection did not lead to differences in PRR responsiveness. This is in contrast to studies where helminth infections were found to influence TLR responses or expression levels [18,34,38,39]. This may be due to the difference between whole blood responses measured here and isolated peripheral blood mononuclear cells used in the other studies. Indeed, a larger follow-up study carried out in the same area measuring cytokine responses in whole blood cultures did not show differences in innate responses (submitted). While whole blood assays are ideal for field conditions as cell separation is not required, their main disadvantage is that the number of cells cultured is unknown and not controlled for. In this study, leukocyte and eosinophil counts did differ significantly between semi-urban and rural Gabonese children, most likely due to the higher rate of *S. haematobium* infection in the rural area. This however did not result in significant differences in innate cytokine responses. Future studies using isolated peripheral blood mononuclear cells and intracellular cytokine staining combined with receptor expression measurements would give more detailed information about differences in immunological responses at the population level.

Our study is one of the few to compare, using identical protocols, not only children in Europe and Africa but also children from rural and semi-urban Africa. The reduced pro-inflammatory response to TLR3 stimulation and enhanced pro-inflammatory response to TLR2/1, TLR2/6 and TLR4 stimulation in African children could affect the efficacy of vaccines or treatments and should be taken into account in future studies. Furthermore, the distinct PRR responses might be of interest when trying to understand differences between populations in terms of allergic and chronic inflammatory diseases.

Materials and methods

Ethics statement

The study was approved in Gabon by the Comité d'Ethique Régional Indépendant de Lambaréné (CERIL N°06/08) and in the Netherlands by the UMC Utrecht Medical Research Ethics Committee. Written informed consent was obtained from parents or legal guardians of all children participating in the study.

Study population

In June-July 2008, 15 schoolchildren were recruited from Lambaréné, a semi-urban municipality in Gabon, and 30 children from the neighboring rural village Zilé in which *Schistosoma haematobium* infection is endemic. Lambaréné [17] and Zilé [18] have been previously described in detail.

S. haematobium infection was determined prior to blood collection by examining a filtrate of 10 mL of urine passed through a 12-µm-pore-size filter (Millipore, Billerica, MA, USA). Children were classified *S. haematobium*-infected when at least one *S. haematobium* egg was detected in the urine, or uninfected when three consecutive urine samples were negative. Infections with intestinal helminths *Ascaris lumbricoides* and *Trichuris trichiura* were determined by analyzing one fresh stool sample using the Kato-Katz method [19]. Hookworm larvae were determined in a 7-day coproculture of the same stool sample [20]. Infection with *P. falciparum* and microfilaria was determined by Giemsa-stained thick blood smears [21].

After collection of blood samples, all *S. haematobium*-infected children were treated with a single dose of praziquantel (40 mg/kg), and those with intestinal helminths received a single dose of albendazole (400 mg) in accordance with the guidelines of the World Health Organization. Children with fungal skin infections received therapy according to local guidelines.

Hematological parameters were analyzed using ADVIA 120 Hematology System (Bayer HealthCare LLC, Diagnostics Division, Tarrytown, NY, USA) and erythrocyte sedimentation rate was measured manually.

In September-October 2008, peripheral blood samples from 21 Dutch children, participating as controls for allergic children in a European study (EuroPrevall, www.europrevall.org), were also collected [22]. No hematological or parasitological data was available for this population, although exposure to helminths and malaria is thought to be unlikely.

Whole blood culture

Heparinized venous blood was processed for culture within 6 hours after venipuncture. Whole blood was diluted 2 times in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin (Astellas Pharma BV, Leiden, the Netherlands), 10 µg/mL streptomycin (Sigma-Aldrich, Saint Louis, MO, USA), 1 mM pyruvate (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich) and stimulated with 50 µg/mL polyinosinic-polycytidylic acid high molecular weight (poly(I:C); InvivoGen, San Diego, CA, USA), 50 ng/mL Pam2CGDPKHPKSF (FSL-1; InvivoGen), 100 ng/mL Pam3CSK4 (Pam3; EMC Microcollections GmbH, Tübingen, Germany), 100 ng/mL ultrapure lipopolysaccharide (LPS; InvivoGen), 10 µg/mL β-D-Glu-mDAP (iE-DAP; InvivoGen), 100 µg/mL mannan (Sigma-Aldrich), 100 µg/mL curdlan (Wako Chemicals GmbH, Neuss, Germany) or 5 ng/mL 1-(palmitoyl)-2-(5-keto-6-octene-dioyl)phosphatidylcholine (KOdiA-PC; Cayman Chemicals, Ann Arbor, MI, USA), alone or in combination (Table 2). Medium was used as a negative control and 2 µg/mL phytohemagglutinin (PHA; Remel, Dartford, UK), a mitogen, as a positive control. 100 µL of ligand(s) in medium was added to wells containing 100 µL of diluted blood in 96-well round-bottom plates (Nunc; Roskilde, Denmark) and incubated in the presence of 5% CO_2 at 37°C for 24 hours. Supernatants were harvested and stored at −80°C.

Table 2. Ligands used in the study, their receptors and adapters.

Ligand	PRR	TLR adapter
Poly(I:C)	TLR3	TRIF
FSL-1	TLR2/6	MyD88
Pam3	TLR2/1	MyD88
LPS	TLR4	MyD88, TRIF
iE-DAP	NLR: NOD1	-
KOdiA-PC	CD36/SR-B2	-
mannan	CLR: MR, DC-SIGN	-
curdlan	CLR: Dectin-1	-

PRR, pattern-recognition receptor. TLR, toll-like receptor. NLR, NOD-like receptor. NOD, nucleotide-binding oligomerisation domain-containing protein 1. SR, scavenger receptor. CLR, C-type lectin receptor. MR, mannose receptor. DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin. TRIF, TIR-domain-containing adapter-inducing interferon-β. MyD88, myeloid differentiation primary response gene 88.

Cytokine analysis

Tumor necrosis factor (TNF) and interleukin-10 (IL-10) levels were measured in supernatants by an enzyme-linked immunosorbent assay (ELISA) according to manufacturers' instructions using half of the reaction volume (Sanquin, Amsterdam, the Netherlands). Levels below detection limit were replaced by half the detection limit, 1.4 pg/mL and 1.2 pg/mL for TNF and IL-10 respectively.

Statistical analysis

Data analysis was performed using IBM SPSS Statistics version 20 for Windows (Armonk, NY, USA: IBM Corp.; 2011). Cytokine concentrations in response to stimulation were corrected for spontaneous cytokine production by subtracting medium responses. Negative values and zeros were subsequently replaced by the lowest positive value, 0.08 and 0.04 for TNF and IL-10 respectively. The pro/anti-inflammatory TNF/IL-10 ratio was calculated by: ($TNF^{stimulated} - TNF^{medium}$) / ($IL\text{-}10^{stimulated} - IL\text{-}10^{medium}$). The degree of interaction between two ligands was calculated for e.g. poly(I:C) and iE-DAP by: ($TNF^{poly(I:C)+iE\text{-}DAP} - TNF^{medium}$) / (($TNF^{poly(I:C)} - TNF^{medium}$) + ($TNF^{iE\text{-}DAP} - TNF^{medium}$)). This could result in a synergistic or inhibitory effect. Mann-Whitney U test and Kruskal-Wallis test with Dunn's post-test were used to compare groups. Fisher's exact test and Pearson chi-square test were used to compare categorical data of the population characteristics. Wilcoxon signed rank test was used to compare the (synergistic) response to combined stimulation with the sum of cytokines produced in two separate stimulations (after background subtraction) and for comparing the response to stimulation with medium condition. p-values less than 0.05 were considered statistically significant.

Graphs were made with GraphPad Prism version 6 for Windows (La Jolla, CA, USA: GraphPad Software; 2013). Box plots have 10-90% range whiskers and bar graphs show medians.

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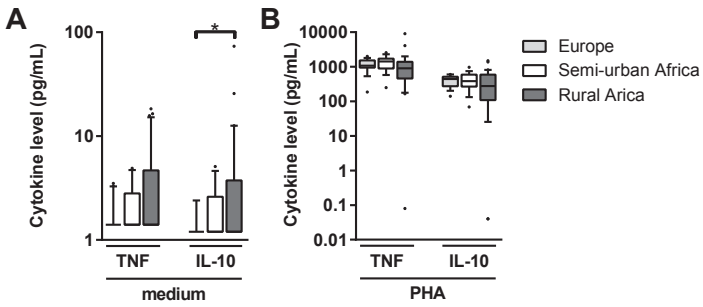
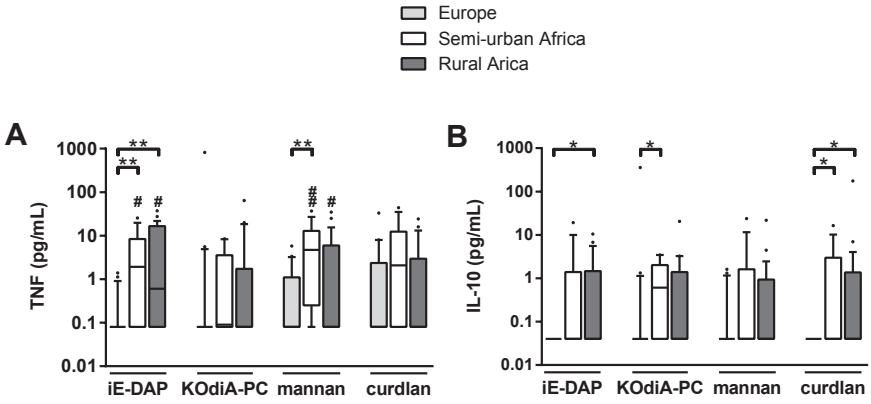
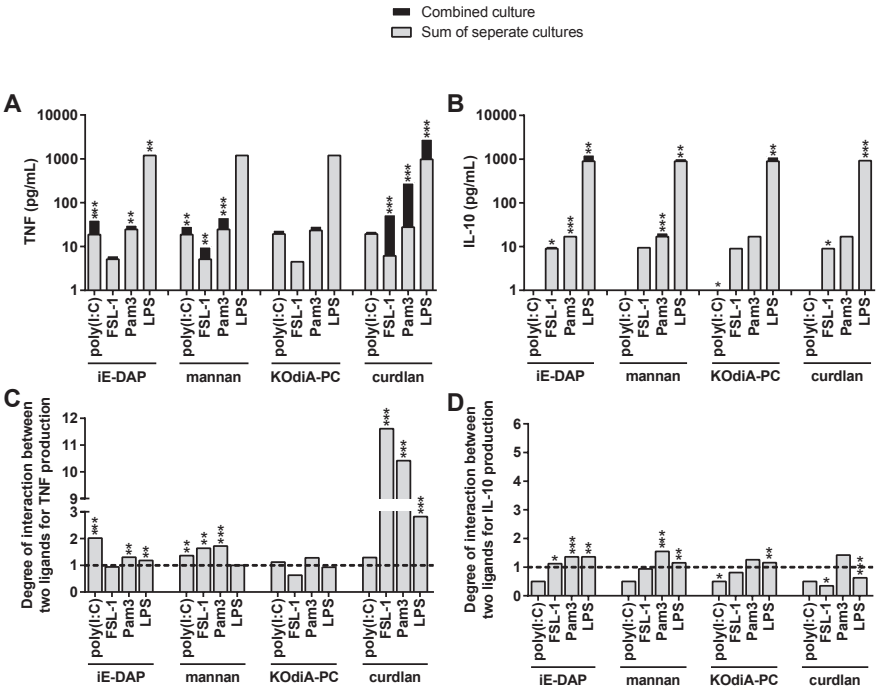
Supplementary material



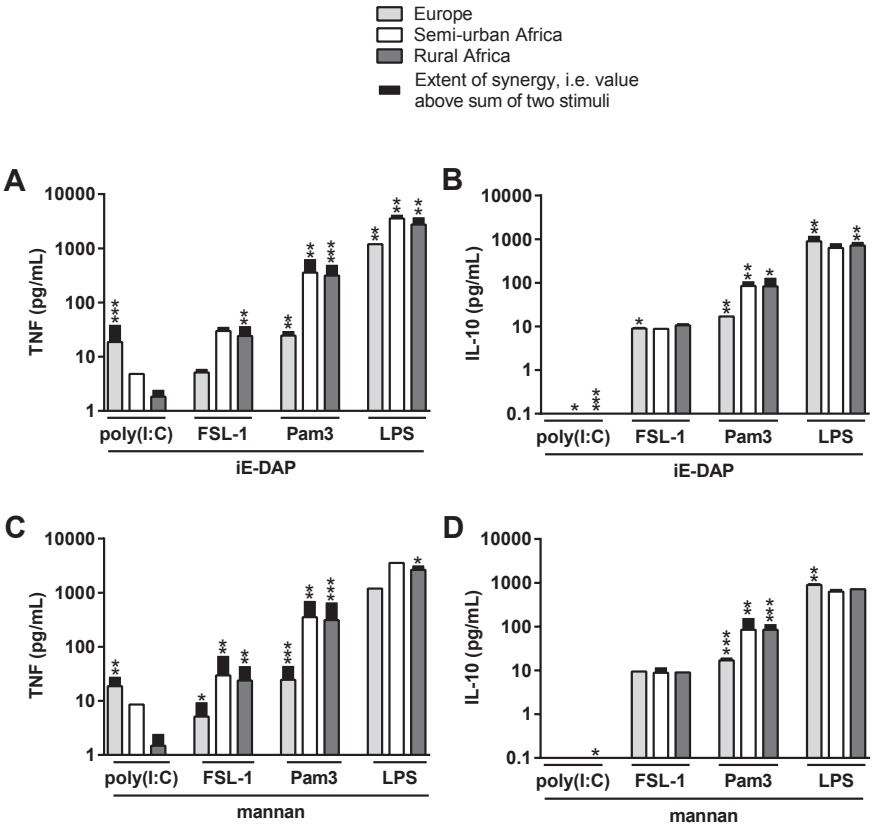
Supplementary Figure 1. Whole blood cytokine production in negative and positive control samples. A) Spontaneous TNF and IL-10 production in negative control samples (medium). B) TNF and IL-10 production in positive control samples (PHA).



Supplementary Figure 2. Whole blood cytokine responses to non-TLR stimulation.
A) TNF production in response to non-TLR ligands (iE-DAP, mannan, KOdiA-PC and curdlan). B) IL-10 response to non-TLR ligands. * p-value when comparing two areas. # p-value when comparing stimulated condition with medium condition.



Supplementary Figure 3. Interaction between TLR and non-TLR ligands in Dutch children.
A) TNF responses to stimulation with poly(I:C), FSL-1, Pam3 or LPS combined with iE-DAP, mannan, KOdiA-PC or curdlan. B) IL-10 responses to combined stimulations. C) Degree of interaction between two ligands for TNF production. D) Degree of interaction between two ligands for IL-10 production.



Supplementary Figure 4. Interaction between TLR and non-TLR ligands.
A) TNF responses to stimulation with poly(I:C), FSL-1, Pam3 or LPS combined with iE-DAP. B) IL-10 responses to TLR ligands combined with iE-DAP. C) TNF responses to TLR ligands combined with mannan. D) IL-10 responses to TLR ligands combined with mannan.

3

Enhanced Pro-inflammatory Cytokine Responses following Toll-Like-Receptor Ligation in *Schistosoma haematobium*-infected Schoolchildren from rural Gabon.

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Abstract

Background: *Schistosoma* infection is thought to lead to down-regulation of the host's immune response. This has been shown for adaptive immune responses, but the effect on innate immunity, that initiates and shapes the adaptive response, has not been extensively studied. In a first study to characterize these responses, we investigated the effect of *Schistosoma haematobium* infection on cytokine responses of Gabonese schoolchildren to a number of Toll-like receptor (TLR) ligands.

Methodology: Peripheral blood mononuclear cells (PBMCs) were collected from *S. haematobium*-infected and uninfected schoolchildren from the rural area of Zilé in Gabon. PBMCs were incubated for 24h and 72h with various TLR ligands, as well as schistosomal egg antigen (SEA) and adult worm antigen (AWA). Pro-inflammatory TNF-α and anti-inflammatory/regulatory IL-10 cytokine concentrations were determined in culture supernatants.

Principal Findings: Infected children produced higher adaptive IL-10 responses than uninfected children against schistosomal antigens (72h incubation). On the other hand, infected children had higher TNF-α responses than uninfected children and significantly higher TNF-α to IL-10 ratios in response to FSL-1 and Pam3, ligands of TLR2/6 and TLR2/1 respectively. A similar trend was observed for the TLR4 ligand LPS while Poly(I:C) (Mda5/TLR3 ligand) did not induce substantial cytokine responses (24h incubation).

Conclusions: This pilot study shows that *Schistosoma*-infected children develop a more pro-inflammatory TLR2-mediated response in the face of a more anti-inflammatory adaptive immune response. This suggests that *S. haematobium* infection does not suppress the host's innate immune system in the context of single TLR ligation.

Introduction

Schistosomiasis is a parasitic disease of major public health importance and is largely chronic in nature. The WHO estimates that more than 207 million people are infected worldwide [1,2]. Chronic helminth infections are generally assumed to cause down-regulation of immune responses allowing long-term survival of the parasite on the one hand and minimizing immune immunopathology on the other [3] with important consequences to the host's health [4–7]. Effects of schistosomal infection on adaptive immunity are widely studied in this context and it has been shown that chronic schistosomiasis inhibits *in vitro* proliferation of human lymphocytes in response to schistosomal antigens [8,9]. The anti-inflammatory cytokine interleukin (IL)-10 plays an important role in this down-regulation [10–14]. Van den Biggelaar and colleagues have demonstrated that children chronically infected with schistosomes produce more IL-10 together with IL-5 and IL-13 in response to adult worm antigen (AWA), than those free of *Schistosoma* infection [15]. Helminth-induced immunomodulation may also have an impact on other infections and vaccine effectiveness. For example, in filarial infections, IL-10 down-regulates responses to unrelated antigens such as tetanus toxoid [16,17]. Similar mechanisms might explain the impaired reaction to tetanus toxoid observed in *Schistosoma mansoni*-infected individuals [18]. Another feature of IL-10 is that it might suppress atopy in *Schistosoma haematobium*-infected children [15,19].

Although it is becoming clearer how chronic schistosomiasis impacts on adaptive immune responses in terms of immunoregulation, the effects of schistosomal infection on innate immunity is a less studied area. In general, the innate immune system detects invading pathogens through a set of 'pattern-recognition receptors' (PRRs) which recognize 'pathogen-associated molecular patterns' (PAMPs) [20,21]. Engagement of PRRs, such as Toll-like receptors (TLRs) initiates early immune events prior to the full activation of adaptive immunity. TLRs act as homo- (e.g. TLR3 or TLR4) or heterodimers (e.g. TLR2/1 or TLR2/6), or in combination with other PRRs such as C-type lectins. Most TLRs induce IL-12p70 which in turn promotes differentiation of Th1 cells and inflammatory responses, characterized by production of tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ). On the other hand, C-type lectins can be in part responsible for the induction of anti-inflammatory responses [22]. The interplay between innate receptors is thought to form the signals that will determine the development of adaptive immune responses [21,23].

The present study is part of the SCHISTOINIR project that aims to explore innate immune responses in schistosomiasis (for further information: www.york.ac.uk/res/schistoinir). This pilot study was set out to investigate how chronic schistosomiasis affects the peripheral blood mononuclear cell (PBMC) response to various TLR ligands. Here, we report the results from children living in a *S. haematobium*-endemic rural area near Lambaréné, Gabon.

Results

Characteristics of the study population

Table 1 shows that the demographic, parasitological and hematological characteristics of the

Table 1. General characteristics of the study population.

	Uninfected	Infected
N	13	17
Age (mean (range))	10.2 y (7-16)	11.4 y (7-16)
Sex (boys / girls)	8/5	9/8
BMI (median (interquartile range))	15.5 kg/m^2 (2.83)	15.7 kg/m^2 (1.64)
S. haematobium infection intensity (geometric mean (range))	Not applicable	9.8 eggs/10ml (1-1002)
Intestinal helminth infection (n/total)	5/12	5/11
N. americanus infection(n/total)	1*/12	2/12
A. lumbricoides infection (n/total)	2**/12	2**/11
T. trichiura infection (n/total)	4* **/12	2**/11
Malaria (n/total)	1/12	0/17
Microfilariasis (n/total)	0/12	1/17
Erythrocyte sedimentation rate (median (range))	15 mm/h (6-50)	15 mm/h (10-88)
Level of white blood cells (median (range))	8.70·10^3/µl (3.80-10.30)	7.20·10^3/µl (5.00-12.40)
Level of lymphocytes (median (range))	3.22·10^3/µl (1.68-5.00)	3.33·10^3/µl (1.26-4.60)
Level of monocytes (median (range))	0.68·10^3/µl (0.36-1.00)	0.64·10^3/µl (0.49-1.04)
Level of neutrophils (median (range))	2.99·10^3/µl (1.06-6.08)	2.14·10^3/µl (1.05-5.79)
Level of eosinophils (median (range))	0.79·10^3/µl (0.20-1.93)	1.53·10^3/µl (0.50-2.49)
Level of basophils (median (range))	0.09·10^3/µl (0.04-0.27)	0.07·10^3/µl (0.04-0.12)
Level of hemoglobin (median (range))	12.2 g/dl (11.4-13.2)	11.3 g/dl (9.1-13.6)

Thick smears (n=1) for the diagnosis of blood parasites as well as stool samples (n=6 for Kato-Katz and n=5 for coproculture) for the diagnosis of intestinal helminths were missing at random. * Including one child with a *T. trichiura - N. americanus* co-infection. ** Including one child with a *T. trichiura - A. lumbricoides* co-infection. *** Including one child with a *T. trichiura - N. americanus* co-infection and another one with a *T. trichiura - A. lumbricoides* co-infection.

infected and uninfected groups of schoolchildren were comparable. Although *S. haematobium*-infected children tended to be more anemic and to have higher eosinophil levels, there were no significant differences between the groups for any of the parameters. In addition, the prevalence and infection intensity of *A. lumbricoides, T. trichiura* or *N. americanus* did not significantly differ between *S. haematobium*-positive and -negative children (data not shown for infection intensity). *S. haematobium* infection intensities were low (<50 epg/10ml) in 14/17 infected children [38]. Malaria and filariasis are endemic in this area [39]. However, only one study participant was infected with *P. falciparum* and one with microfilariae.

Cytokine responses to TLR-ligands

TLR-induced cytokine production by PBMC cultures from infected and uninfected groups was measured at 24h (Figure 1A, upper panel). While Pam3, FSL-1 and LPS led to substantial and significant cytokine production in both groups compared to medium control, the TLR3 ligand poly(I:C) did not stimulate significant levels of TNF-α or IL-10. Pam3, FSL-1 and LPS tended to induce the production of greater quantities of the pro-inflammatory cytokine TNF-α in infected compared to uninfected children (Pam3; $p<0.01$), while the production of IL-10 was comparable between the two groups.

There was a stronger pro-inflammatory index for PBMC responses of infected children (Figure 1B, upper panel) upon TLR-stimulation (except poly(I:C)) than those from uninfected children. These differences were significant for both Pam3 and FSL-1 ($p<0.05$) which are ligands for TLR2.

Furthermore, *S. haematobium* infection intensity was positively associated with TLR-2- and TLR-4-mediated TNF-α but not with IL-10 responses. This was significant for Pam3 ($\rho = 0.80$) and FSL-1 ($\rho = 0.66$). Also, pro-inflammatory indices tended to be positively associated with infection intensity (significant for Pam3 with $\rho = 0.60$).

In order to get an idea of the dynamics of the innate response, TLR responses were also measured after 72h. Cytokine responses to TLR ligands decreased over time, being less pronounced after 72h. Nevertheless, the difference between infected and uninfected subjects was similar to that observed in 24h cultures with a largely pro-inflammatory TLR response in infected subjects (Figure 1, lower panels).

Cytokine responses to schistosomal products

We examined adaptive immune responses by analyzing cytokine production by PBMCs after 72h of incubation with schistosomal antigens (Figure 2, lower panels). Since these products not only contain antigenic components but also innate ligands [40–47], cytokine production was assessed at both 24h (innate) and at 72h (adaptive response).

SEA- and AWA-induced IL-10 was detected in PBMC culture supernatants after 24h and increased further in 72h cultures. IL-10 production in infected children was consistently higher than in uninfected children at 24h but this difference was only significant at 72h: both SEA- and AWA-induced IL-10 responses were significantly higher in infected children at 72h (Figure 2A).

After 24h, AWA-stimulated cultures from infected children secreted significant levels of TNF-α ($p<0.05$ cf. medium), while in uninfected children, antigen-induced TNF-α was only

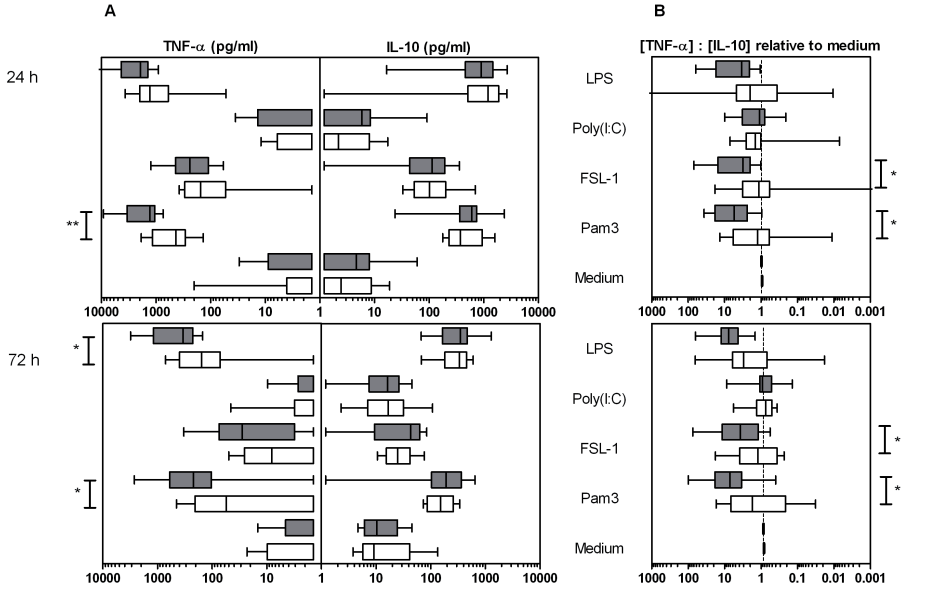

Figure 1. Cytokine production in response to TLR ligands in PBMC cultures.
White and grey boxes correspond to *S. haematobium*-free and infected children respectively with the whiskers indicating minimal and maximal concentrations. * $p < 0.05$; ** $p < 0.01$. Panel A: At 24h, the 'innate' time-point, infected children produced significantly more TNF-α in response to the TLR2/1 ligand Pam3CSK4 (Pam3) compared with uninfected children. TLR-mediated IL-10 production did not significantly deviate between infection groups. When innate cytokine responses faded at 72h, similar trends were observed. These plots were not adjusted for spontaneous cytokine production. Panel B: At both time points, pro-inflammatory indices (i.e. cytokine ratio induced by one of the stimuli relative to the spontaneously produced ratio) induced by the TLR2 ligands, Pam3 and FSL-1, were significantly higher in infected versus uninfected children.

detected after 72h.

After 24h, the cytokine ratios following stimulation with schistosomal products seemed more anti-inflammatory than pro-inflammatory (i.e. increased IL-10 : TNF-α; Figure 2B, upper panel). After 72h, the pro-inflammatory index tended to increase in uninfected children, and was significantly higher than in infected children upon stimulation with AWA (Figure 2B, lower panel).

Discussion

In this pilot study we investigated the effects of chronic *Schistosoma* infection on TLR-mediated cytokine production. We showed that innate TNF-α responses and TNF-α : IL-10 ratios upon TLR2 stimulation of PBMCs were significantly higher in *S. haematobium*-infected children compared with those without infection, in the face of enhanced regulatory adaptive responses to schistosomal antigens. This suggests that schistosomal infection is associated with elevated

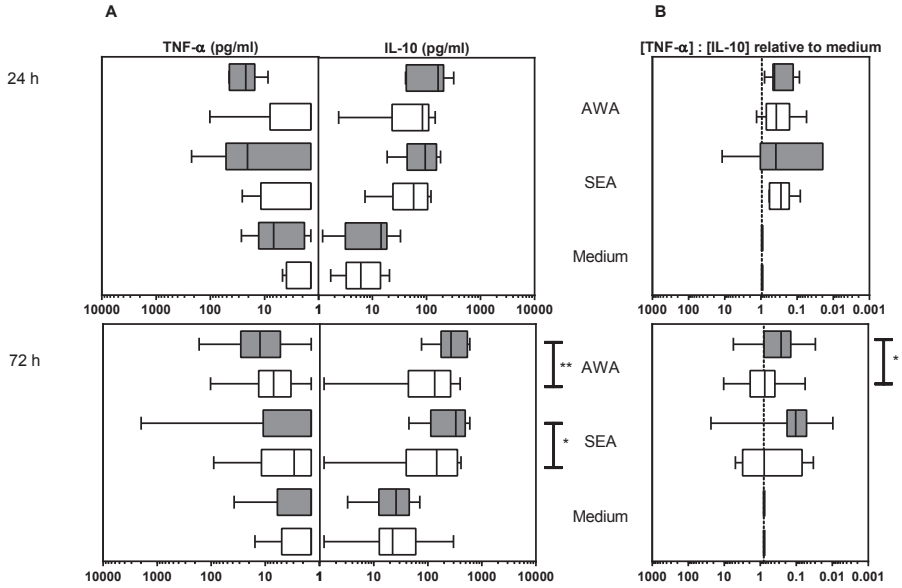

Figure 2. Cytokine production in response to schistosomal products in PBMC cultures.
PBMCs were stimulated with schistosomal egg antigen (SEA) or adult worm antigen (AWA). White and grey boxes correspond to *S. haematobium*-free and infected children respectively with the whiskers indicating minimal and maximal concentrations. * $p < 0.05$; ** $p < 0.01$. Panel A: Cytokine production did not differ between groups at 24h (mainly innate response) but the adaptive IL-10 response after 72h of incubation to both schistosomal products was significantly higher in infected children than in *S. haematobium*-free children. Furthermore, only infected children produced a significant innate TNF-α response to schistosomal products at 24h. These plots are not adjusted for spontaneous cytokine production. Panel B: After 24h (predominantly innate), most cytokine ratios induced by schistosomal products were more anti-inflammatory than the ratios induced by medium alone. When the adaptive response had developed after 72h however, only infected children produced significant anti-inflammatory cytokine balances while pro-inflammatory indices were lower in infected children than in uninfected children (AWA; $p < 0.05$).

pro-inflammatory TLR2 responses.

While cytokine responses upon TLR stimulation tended to decrease over time, the reverse was seen after stimulation with schistosomal products. Although at 24h, TNF-α levels tended to be more pronounced in infected than in uninfected children, analogous with the observed TLR-mediated cytokine responses, after 72h of stimulation, PMBCs from *S. haematobium*-infected children produced significantly more IL-10 than uninfected children, as has been described elsewhere [15]. Schistosomal products are more complex than single TLR stimuli. SEA and AWA stimulate the innate immune system through TLRs, C-type lectins and other innate receptors [40–47] but in addition, they contain antigens which can be processed and presented to the T cell receptor forming the basis of acquired immune responses. Schistosomal products are thus able to activate both innate and adaptive pathways. This may explain the observed trend of an initial pro-inflammatory-like immune response. Concurrent with a fading innate cytokine response, a clear anti-inflammatory adaptive response comprising elevated IL-10 was detected

in infected children at 72h.

To our knowledge there are only two other human studies on TLR-mediated cytokine profiles in schistosomiasis. Van der Kleij et al. reported reduced TLR responses in Gabonese *S. haematobium*-infected as compared to uninfected children [24]. These results seem to contradict the current findings, but can be explained by differences in the selection of the uninfected control groups. While in the present study both infected and uninfected children were from the same *S. haematobium*-endemic rural area (Zilé), Van der Kleij et al. recruited the uninfected control group from a non-endemic neighboring semi-urban area to ensure that the negative subjects were truly negative with no history of exposure. However, when we examined the dataset of the Van der Kleij study to compare the infected (n=5) and uninfected (n=10) groups from the same rural area of Zilé, infected children tended to produce higher TNF-α levels as well as higher pro-inflammatory indices in response to LPS than their uninfected counterparts, which is in line with our results.

The second study of TLR-mediated cytokine profiles was carried out in Brazil and also corresponds with our results. Although they did not highlight it, Montenegro et al. showed that 48h whole blood cultures from *S. mansoni*-infected adults produced more TNF-α than those of uninfected adults in response to LPS [48].

In addition, a mouse model of infection pointed towards a similar association between TLR function and schistosomal infection and suggests that schistosomal infection induces elevated pro-inflammatory TLR responses [49].

Few field studies on TLR responses in other helminth infections than schistosomiasis have been published and show varying results. In contrast to *S. haematobium* infection, a negative association has been found between infection with the filarial nematode *Wuchereria bancrofti* and intracellular pro-inflammatory cytokine expression upon TLR stimulation in lymphocytes and monocytes in a population in South India [50,51]. In addition, *W. bancrofti* was associated with reduced pro-inflammatory cytokine responses upon TLR stimulation of PBMCs in a population from the same area with latent tuberculosis [52]. On the other hand, Jackson et al. showed infections with intestinal nematodes, i.e. hookworm and *Trichuris trichiura*, to be associated with pro-inflammatory cytokine production, but not with regulatory cytokine production, upon TLR2 and TLR4 stimulation of monocytes from children from Pemba Island [53]. Apparently, the effect of helminth infections on the innate immune responses is species specific; some helminth species may be associated with more pro-inflammatory TLR responses while other helminths are associated with more anti-inflammatory TLR responses. In the present study, *S. haematobium*-infected and uninfected children did not significantly differ with respect to the number and species of other helminth infections. However, information on soil-transmitted helminth infections was not available for some of the participants and we cannot entirely rule out that these infections may have altered the observed innate cytokine responses. In addition, other parasites such as malaria may influence immune balances [54]. The duration as well as the intensity of infection might further influence innate immune responses. It is therefore important to confirm our findings in a larger study population and in varying epidemiological settings.

In conclusion, this pilot study on innate immune responses in schistosomiasis shows a

more pro-inflammatory response to single TLR2 ligands in the face of an anti-inflammatory adaptive immune response in *S. haematobium*-infected children. Whilst the precise biological mechanisms for these observations remain to be ascertained, it seems that the commonly accepted view that schistosomal infection suppresses the host's immune system does not hold for ligation of single TLRs. Many receptors – TLRs as well as non-TLRs such as C-type lectins – are involved in innate signaling and their interactions as well as down-stream pathways are at the basis of the immune response to invading pathogens [21,55]. Given the fact that a *Schistosoma* worm is a complex mix of ligands stimulating the innate immune system, further research into this innate cross-talk is necessary.

Materials and methods

Ethics statement

The study was approved by the "Comité d'Ethique Regional Independent de Lambaréné" (CERIL). Written informed consent was obtained from parents or legal guardians of all children participating prior to inclusion into the study.

Study site and population

This pilot study was performed in Zilé, a rural area situated 16 km from Lambaréné, Gabon [24,25]. This area is endemic for *S. haematobium*, but with important focal differences. Within Zilé there are two smaller communities, one with high (PK15) and one with low transmission (PK17), 2 km apart from each other. In June and July 2008, we recruited 17 *S. haematobium*-infected schoolchildren from PK15 and 13 uninfected from PK17, and compared their immune profiles. Study participants had no history of anti-helminthic treatment within the last 3 months prior to this study.

Parasitological examination

S. haematobium infection was determined a maximum of seven days prior to blood collection by examining the residue of 10 ml of urine passed through a filter of 12-µm pore-size (Millipore). Children were classified as *S. haematobium*-infected if at least one *S. haematobium* egg was detected in the urine, or uninfected if three consecutive urine samples were negative. Geometric mean infection intensity was calculated for infected children using the first urine reading. Infections with intestinal helminths *Ascaris lumbricoides* and *Trichuris trichiura* were determined by analyzing one fresh stool sample using the Kato-Katz method 30-45 minutes after preparation [26]. *Necator americanus* larvae were detected in a 7-day coproculture of the same stool sample. To this end the classical charcoal culture procedure was used [27–29]. Infection with *Plasmodium falciparum* and microfilaria was determined by Giemsa-stained thick blood smears [30].

After collection of blood samples, all *S. haematobium*-infected children were treated with a single dose of praziquantel (40 mg/kg), and those with intestinal helminths were given a single dose of albendazole (400 mg). Children with other infections or clinical complaints were provided with appropriate medical care.

Hematology

Hematological parameters were analyzed using the ADVIA® 120 Hematology System (Bayer Health Care) and erythrocyte sedimentation rate was determined manually.

PBMC cultures

PBMCs were isolated from heparinized venous blood by density centrifugation on Ficoll (AZL pharmacy, The Netherlands) and cultured in 200 µl of IMDM supplemented with 5% fetal bovine serum (Greiner Bio-One), 100 U/ml penicillin (Astellas), 10 µg/ml streptomycin, 1 mM pyruvate and 2 mM L-glutamine (Sigma) in 96-well round bottom plates (Nunc). PBMCs were seeded at 1 x 10^6 cells/well for stimulation with schistosomal egg antigen (SEA) or adult worm antigen (AWA), and at 5 x 10^5 cells/well for stimulation with specific TLR ligands and phyto-hemagglutinin (PHA; positive control). IMDM medium was used as a negative control. Supernatants were collected after 24h and 72h of incubation at 37°C in a 5% CO_2 atmosphere and stored at -80°C until cytokine analysis.

Innate and adaptive stimuli

A panel of four TLR ligands was used:

1. TLR2/1 ligand, Pam3CSK4 (Pam3), a synthetic bacterial triacylated lipopeptide (final concentration of 100 ng/ml; EMC microcollections GmbH),
2. TLR2/6 ligand, FSL-1, a synthetic diacylated lipoprotein mimicking the N-terminal part of LP44 from Mycoplasma salivarium (50 ng/ml; InvivoGen),
3. TLR3 ligand polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of viral double-stranded RNA (50 µg/ml; InvivoGen),
4. TLR4 ligand, ultrapure lipopolysaccharide (LPS) derived from Escherichia coli (100 ng/ml; InvivoGen).

In addition to the classical innate ligands, we used the schistosomal antigen-containing products SEA and AWA from *S. haematobium* at a final concentration of 10 µg protein/ml. Purified PHA (Remel) was used as a positive control at a final concentration of 2 µg/ml.

Cytokine analysis

Two cytokines were measured in this pilot study; one key pro-inflammatory cytokine, TNF-α [31–33], and the anti-inflammatory/regulatory cytokine IL-10 [12,34–37]. Cytokine concentrations were measured in supernatants from single PBMC cultures by ELISA according to the manufacturer's instructions, using half of the reaction volume (PeliKine CompactTM, Human TNF-α and IL-10 ELISA kit, Sanquin).

A pro-inflammatory index was computed as the TNF-α : IL-10 ratio upon stimulation relative to the spontaneously produced TNF-α : IL-10 ratio (i.e. in medium):

$$([\text{TNF-}\alpha]_{\text{stimulus A}}/[\text{IL-10}]_{\text{stimulus A}})/([\text{TNF-}\alpha]_{\text{medium}}/[\text{IL-10}]_{\text{medium}})$$

Statistical analysis

Differences between schistosome-infected and uninfected groups were determined by the Fisher's exact test for sex and intestinal helminth infections and by the Mann-Whitney U test for helminth infection intensity, body mass index (BMI) and hematological parameters. Age was normally distributed and differences between infection groups were tested using the independent student's T test. As cytokine concentrations were not normally distributed, nonparametric tests were used. Differences in an individual's cytokine response to different stimuli (e.g. medium versus stimulus-induced cytokine concentration, or ratio) were tested by the Wilcoxon matched-pair signed-rank test.

Differences between infection groups in cytokine concentrations and pro-inflammatory indices were tested by the Mann-Whitney U test. Cytokine concentrations were corrected for spontaneous cytokine production. This was done by subtracting the spontaneously induced cytokine concentration (i.e. in medium) from the stimulus-induced cytokine concentration. For the pro-inflammatory index, this was done by dividing the stimulus-induced ratio by the spontaneously induced ratio (see formula above). Similarly, Spearman's ρ was calculated to estimate the correlation between cytokine responses and infection intensity.

SPSS 18.0 (SPSS Inc.) and GraphPad Prism 5 (GraphPad Software, Inc.) were used for statistical analysis. Results were considered significant when the p-value was <0.05.

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4

Praziquantel treatment shows that down-regulation of antigen specific immune responses in human schistosomiasis is associated with $CD4^+CD25^+FOXP3^+$ T cells.

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Author Summary

Schistosomiasis is a chronic infection that affects over 200 million people in particular children in developing countries. To answer the question how infection affects the immune system, we conducted a longitudinal study in schoolchildren with and without *Schistosoma haematobium* infection before and after treatment with praziquantel living in an endemic area in Gabon. We used whole blood assays to investigate innate immune responses, by stimulating pattern recognition receptors, and adaptive immune responses, by stimulating with schistosomal antigens. We also characterized the $CD4^+$ T cell memory compartment and assessed the frequency of $CD4^+CD25^+FOXP3^+$ T cells. After removal of *S. haematobium* infection by praziquantel, the most prominent effect was seen on parasite antigen-specific cytokine responses. An increase was seen in schistosome-specific antigen responses following treatment, which was inversely associated with a decrease in $CD4^+CD25^+FOXP3^+$ T cells. These results for the first time provide evidence for the association between schistosome infection, regulatory T cells and antigen specific immune hypo-responsiveness in humans.

Abstract

Background: *Schistosoma haematobium* infection results in alterations in immune function, yet the concurrent interplay between the various arms of the immune system and the effect of parasite removal following anthelmintic treatment has not been studied extensively. We conducted a longitudinal study to investigate the effect of *S. haematobium* infection and its clearance on innate and adaptive immune responses, on $CD4^+$ T cell memory compartment and on $CD4^+CD25^+FOXP3^+$ T cells.

Methods: Cytokine responses of Gabonese schoolchildren infected with *S. haematobium* (n=40) were studied in whole blood at pre- and 7 months post-praziquantel (PZQ) treatment; responses of uninfected children (n=39) were studied at the two time points as controls. To assess adaptive immune responses, the production of interferon gamma (IFN-γ), interleukin-2 (IL-2), IL-5, IL-10 and tumor necrosis factor (TNF) was measured after stimulation with schistosome soluble egg and adult worm antigens and the mitogen PHA. Innate immune responses were assessed by the production of IL-10, IL-1β, IL-1ra and TNF following culture with Pam3, mannan and curdlan, alone or in combination. Cytokines in supernatants were quantified by Luminex. Flow cytometry was used to characterize the memory T cell compartment and to assess $CD4^+CD25^+FOXP3^+$ T cell frequencies.

Results: Schistosome-specific responses increased in *S. haematobium*-infected schoolchildren following treatment with PZQ, but the effect of removal of infection on innate immune responses was marginal. Furthermore PZQ treatment resulted in increased effector memory T cells (T_{EM}) in infected schoolchildren. $CD4^+CD25^+FOXP3^+$ T cell frequencies, which were higher in infected children, decreased following anti-schistosome treatment. In *S. haematobium*-infected children there was a significant negative association between the decrease in $CD4^+CD25^+FOXP3^+$ T cell frequencies and the increase in schistosome-specific IL-5 and IL-10 cytokine levels observed upon treatment.

Conclusions: This study supports the notion that schistosome infections in humans can lead to the expansion of $CD4^+CD25^+FOXP3^+$ T cells leading to down-regulation of schistosome-specific immune responses.

Introduction

Schistosomiasis, caused by the parasitic trematodes of the genus *Schistosoma*, affects over 200 million people worldwide, especially children [1]. Chronic infections with schistosomes are associated with immune hypo-responsiveness characterized by reduced in vitro proliferation of T lymphocytes and decreased Th1- and Th2-type cytokine production in response to not only schistosomal antigens [2] but also to third party antigens such as Mycobacterium tuberculosis purified protein derivative (Mtb PPD) [3] and the influenza vaccine [4]. Treatment with antischistosomal drug praziquantel leads to clearance of infection [5] and elevated antigen-specific proliferation [6] and cytokine production [7,8]. The mechanisms of T cell hypo-responsiveness during chronic schistosomiasis are not well understood, but a number of recent reports have shown that in addition to impaired dendritic cell activity during schistosome infection [9], regulatory T cells [10] and regulatory B cells [11] are expanded in schistosome-infected compared to uninfected individuals. However, few studies have examined the effect that parasite elimination by chemotherapy has on $CD4^+CD25^+FOXP3^+$ T cells and how this is linked to effector T cell responses.

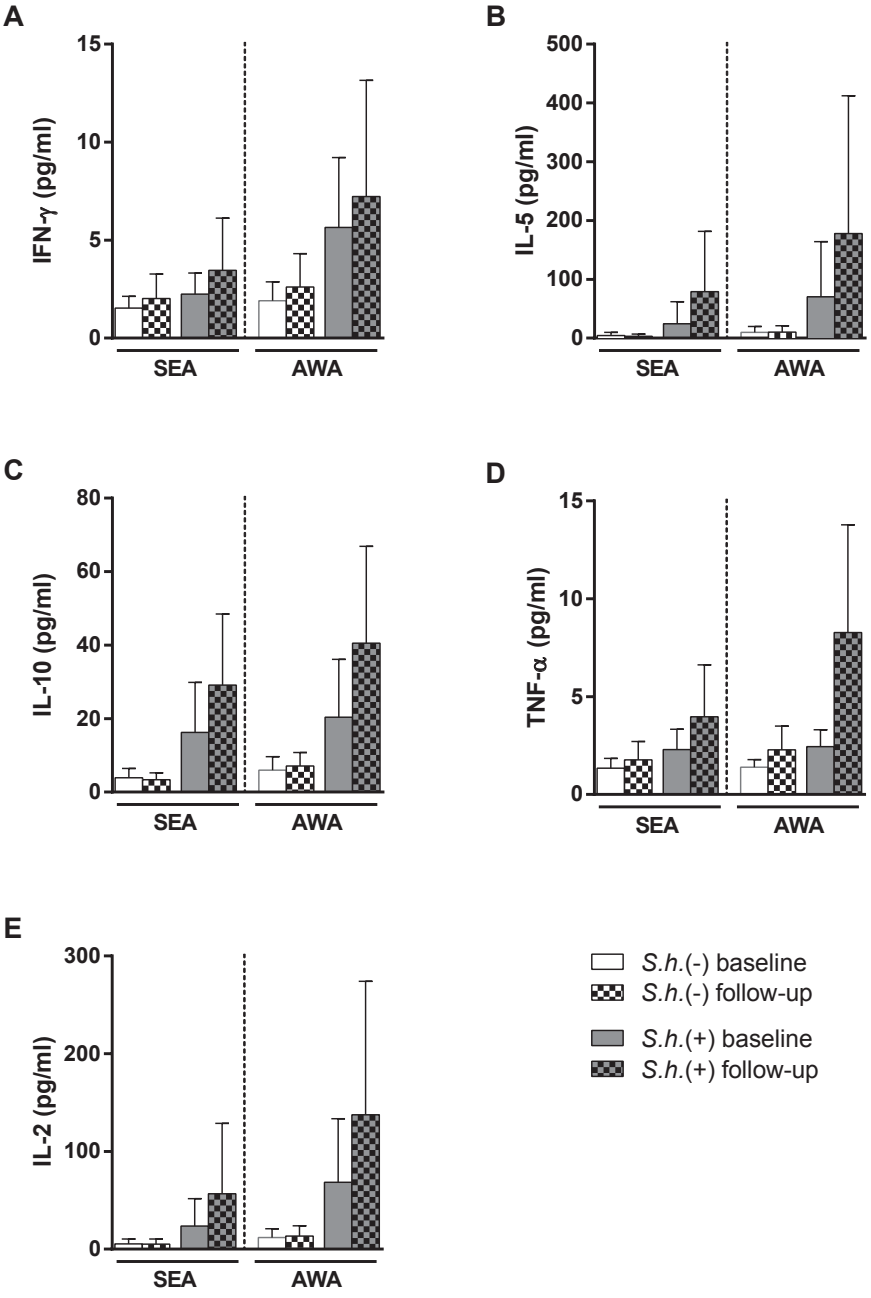
Moreover there are very few studies on innate immune responses in the context of human schistosomiasis. Responses to and expression levels of the Toll-like receptors (TLRs) have been shown to be altered in *Schistosoma haematobium*-infected individuals. For example, myeloid dendritic cells (mDCs) from infected individuals had reduced capacity to respond to TLR ligands [9], PBMCs from infected children produced higher levels of TNF in response to TLRs [12], but TLR2 expression was significantly reduced in children infected with schistosomes compared to uninfected age-matched individuals [13]. Another class of pattern recognition receptors (PRRs), the C-type lectins (CLRs) which includes DC-SIGN and mannose receptor (MR), has also been shown to be important in the recognition of schistosomal ligands [14]. It has been proposed that innate immune responses could be fine-tuned via interaction between distinct PRRs [15,16]. For example schistosomal glycolipids activate DCs to produce inflammatory cytokines through the cooperation of TLR4 and DC-SIGN [17]. However, altogether, little is known about innate immune responses in humans during the course of schistosome infection and the data available come from studies that were cross-sectional in design.

To our knowledge there are no reports that have examined, within one study, the effect of schistosome infection on different components of the immune system. Here we have conducted a longitudinal study where both adaptive and innate immune responses were examined in *S. haematobium*-infected individuals before and after treatment with praziquantel. To this end, we have analyzed cytokine responses to schistosomal antigens as well as to stimuli that trigger PRRs. In order to assess how schistosome infection affects the different components of the adaptive immune system, we have characterized the $CD4^+$ T cell memory compartment and have determined the frequency of $CD4^+CD25^+FOXP3^+$ T cells during infection and after removal of schistosomes by chemotherapy.

Table 1: Characteristics of the study population.

	S. haematobium uninfected	*S. haematobium* infected	p value
Participants (n)	39	40	
Mean age in years (range)	11.31 (6 - 15)	12.73 (9 - 20)	0.006[a]
Sex (male/female)	24/15	17/23	0.117[b]
P. falciparum infection (n/total)	19/39	18/40	0.823[b]
Intestinal helminth infection (n/total)	30/39	32/39	0.780[b]
A. lumbricoides infection (n/total)	17/39	15/39	0.818[b]
T. trichiura infection (n/total)	24/39	24/39	1.000[b]
Hookworm infection (n/total)	14/39	15/39	1.000[b]
Erythrocyte sedimentation rate (mm/h): mean (95% CI)	23.14 (6.70 - 57.25)	20.10 (2.00 - 51.00)	0.372[a]
Level of white blood cell ($x10^3$/µL): mean (95% CI)	8.39 (4.69 - 13.02)	9.12 (6.10 - 13.84)	0.182[a]
Level of lymphocytes ($x10^3$/µL): mean (95% CI)	3.20 (1.51 - 4.59)	3.42 (2.12 - 5.28)	0.271[a]
Level of monocytes ($x10^3$/µL): mean (95% CI)	0.84 (0.36 - 2.00)	0.76 (0.47 - 1.45)	0.332[a]
Level of neutrophils ($x10^3$/µL): mean (95% CI)	2.80 (1.07 - 4.74)	2.72 (1.74 - 3.82)	0.765[a]
Level of eosinophils ($x10^3$/µL): mean (95% CI)	1.09 (0.13 - 2.55)	1.37 (0.42 - 3.02)	0.174[a]
Level of basophils ($x10^3$/µL): mean (95% CI)	0.07 (0.03 - .15)	0.07 (0.03 - .14)	0.707[a]
Level of platelets ($x10^3$/µL): mean (95% CI)	255.17 (106.50 - 374.10)	245.92 (113.40 - 420.00)	0.623[a]
Level of haemoglobin (g/dL): mean (95% CI)	11.89 (10.50 - 13.34)	11.78 (10.35 - 13.68)	0.597[a]

CI, confidence interval. Infections are depicted as number of participants infected out of total number of participants tested. [a]Independent student's T-test; [b]Fisher's Exact Test.


A
IFN-γ (pg/ml)
15
10
5
0
SEA
AWA
B
IL-5 (pg/ml)
500
400
300
200
100
0
SEA
AWA
C
IL-10 (pg/ml)
80
60
40
20
0
SEA
AWA
D
TNF-α (pg/ml)
15
10
5
0
SEA
AWA
E
IL-2 (pg/ml)
300
200
100
0
SEA
AWA
S.h.(-) baseline
S.h.(-) follow-up
S.h.(+) baseline
S.h.(+) follow-up

Results

Study population characteristics

We recruited *S. haematobium*-infected (N = 40) and -uninfected (N = 39) schoolchildren; Table 1 shows the demographic, parasitological and hematological characteristics of the two study groups. There were no significant differences between these two groups in the prevalence of *P. falciparum*, *A. lumbricoides*, *T. trichiura* and hookworm or in hematological parameters at baseline. Similarly, the number of females and males was comparable but infected children were approximately one year older. Schistosome infection parameters as assessed by egg counts, plasma CAA, hematuria and RSI score are given in Table 2; and as expected all were significantly correlated with each other ($p < 0.001$). Praziquantel treatment significantly reduced all infection parameters in the infected schoolchildren ($p < 0.001$; Table 2).

Cytokine responses to schistosomal antigens and mitogen

Age-adjusted mean cytokine levels following whole blood stimulation with SEA and AWA before and 7 months after praziquantel treatment are shown in Figure 1 and Table 3. At baseline, *S. haematobium* infected schoolchildren responded to SEA and AWA, by producing IFN-γ,IL-2, IL-5, IL-10 and TNF while uninfected controls did not (Figure 1). There were no differences in response to PHA between infected and uninfected controls. Following removal of worms by treatment, cytokine responses were enhanced. Specifically, IL-5, IL-10 and IL-2 levels in response to SEA and IL-5, IL-10 and TNF levels in response to AWA were significantly increased in *S. haematobium*-infected children (Figure 1 and Table 3), as was IL-10 in response to PHA ($p=0.001$). No differences were observed in the uninfected control children between baseline and 7 month follow-up time point. In addition, no differences were seen in spontaneous cytokine production (in unstimulated cultures) between infected and uninfected children or between pre- and post-treatment responses (Supplementary figure 1B).

Cytokine responses to TLR and CLR ligands

There were no differences in cytokine responses to Pam3 (TLR1/2 ligand) between *S. haematobium*-infected schoolchildren and uninfected controls at baseline (Figure 2). Over the 7 month study period, the levels of IL-1β and IL-10 decreased significantly in all study subjects, which might reflect the decrease that was seen in *P. falciparum* infection and/or infection load in both *S. haematobium* infected (baseline 18/40 infected vs. follow-up 10/38 infected; $p=0.011$) and uninfected controls (baseline PCR ct value 39.25 vs. follow-up 35.34; $p=0.024$)

Figure 1: Cytokine production in response to schistosomal antigens in whole blood cultures at baseline and 7 months follow-up.

Whole blood from *S. haematobium* infected schoolchildren and uninfected controls was stimulated with schistosomal egg antigen (SEA) and adult worm antigen (AWA). After 72 hours of culture levels of Th1 (A), Th2 (B), regulatory (C), pro-inflammatory (D) and IL-2 (E) cytokines were measured by Luminex. The follow-up samples were obtained at 7 months post-treatment with praziquantel of children that were infected with *S. haematobium* at pre-treatment. The uninfected controls were also examined at the same follow-up period but did not receive praziquantel.

Table 2: Median values of infection indices in the study population.

	Baseline		Follow-up	
	S. haematobium uninfected	*S. haematobium* infected	*S. haematobium* uninfected	*S. haematobium* infected
Median egg counts (IQR)	0.0 (0.0 – 0.0)[a]	19.5 (5.3 – 87.0)[a,b]	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)[b]
Plasma CAA levels pg/ml (IQR)	0.0 (0.0 – 3.0)[a]	420.5 (12.3 – 1196.3)[a, b]	0.0 (0.0 – 0.3)	12.5 (0.0 – 74.3)[b]
Haematuria score (IQR)	0.0 (0.0 – 0.0)[a]	3.0 (0.0 – 3.0)[a, b]	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)[b]
RSI score (IQR)	0.0 (0.0 – 2.0)[a]	5.0 2.0 – 6.3)[a, b]	0.0 (0.0 – 2.0)	0.0 (0.0 – 1.0)[b]

CAA, circulating anodic antigen; RSI, reagent strip index; IQR, interquartile range. Differences between groups were tested with Mann-Whitney U test and within group differences between baseline and follow-up with Wilcoxon matched pairs test. Significant between group differences are indicated with [a] and within group differences with [b]; all p<0.001. Infection indices were all significantly correlated with each other: egg counts with hematuria ($\rho = 0.767$, $p < 0.001$); egg counts with RSI ($\rho = 0.746$, $p < 0.001$); CAA with hematuria ($\rho = 0.632$, $p < 0.001$); CAA with RSI ($\rho = 0.556$, $p < 0.001$); and egg counts with CAA ($\rho = 0.748$, $p < 0.001$).

Table 3: Changes in cytokine production in response to schistosomal products in whole blood cultures 7 months after praziquantel treatment.

		S. haematobium uninfected		*S. haematobium* infected	
Cytokine	Stimulus	Post-/Pre-tx ratio (95% CI)	p value	Post-/Pre-tx ratio (95% CI)	p value
IFN-γ	SEA	1.25 (0.78 - 2.00)	0.332	1.67 (1.00 - 2.78)	0.051
	AWA	1.32 (0.82 - 2.14)	0.243	1.34 (0.71 - 2.51)	0.339
IL-5	SEA	0.69 (0.28 - 1.71)	0.408	3.60 (1.92 - 6.76)	0.001[a]
	AWA	1.03 (0.56 - 1.90)	0.922	2.67 (1.78 - 4.00)	<0.001[a]
IL-10	SEA	0.81 (0.45 - 1.47)	0.476	2.00 (1.34 - 2.98)	0.002[a]
	AWA	1.16 (0.63 - 2.11)	0.623	2.07 (1.41 - 3.03)	0.001[a]
TNF	SEA	1.25 (0.85 - 1.84)	0.235	1.84 (1.08 - 3.14)	0.027
	AWA	1.65 (1.16 - 2.37)	0.008	3.34 (1.65 - 6.76)	0.002[a]
IL-2	SEA	0.90 (0.46 - 1.76)	0.758	2.61 (1.68 - 4.07)	<0.001[a]
	AWA	1.11 (0.57 - 2.16)	0.744	2.03 (1.14 - 3.61)	0.020

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; SEA, schistosomal egg antigen; AWA, adult worm antigen; tx, treatment. Differences within groups were tested with a paired samples T test and the resulting mean differences were anti-log-transformed to obtain the before/after treatment ratio. [a]significant change after α-adjustment by the Bonferroni-Holm method.

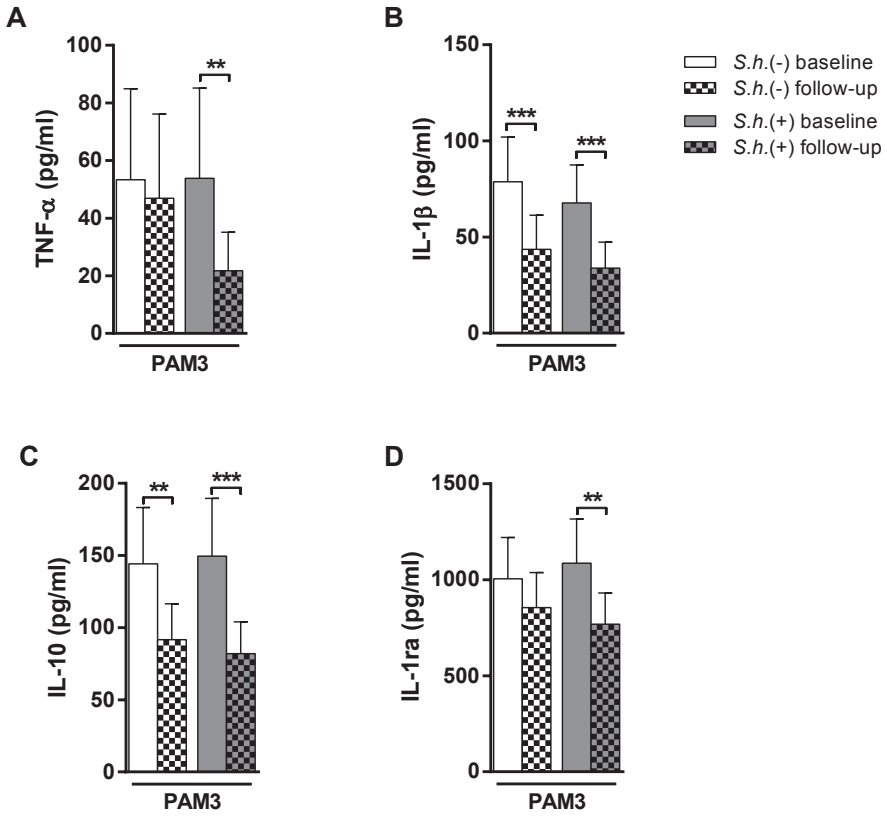


Figure 2: Cytokine production in response to TLR2/1 stimulation in whole blood cultures at baseline and 7 months follow-up.

Whole blood from *S. haematobium* infected schoolchildren and uninfected controls was stimulated with Pam3 (TLR2/1). After 24 hours of culture TNF (A), IL-1β (B), IL-10 (C) and IL-1RA (D) were measured by Luminex. Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as age-adjusted geometric means. * p< 0.05, ** p< 0.01, *** p< 0.001.

over time. However, TNF response to Pam3 decreased significantly, after treatment of *S. haematobium* infected subjects, a response that was different from that observed in uninfected controls, indicating that removal of *S. haematobium* infection is associated with decrease in TNF production in response to Pam3. Responses to mannan (DC-SIGN/MR ligand) and curdlan (Dectin-1 ligand) alone were largely not detectable (data not shown). However when combined with Pam3, significant synergy for pro-inflammatory responses (TNF and IL-1β) was observed. For anti-inflammatory/regulatory responses (IL-10 and IL-1ra) combined stimulation of Pam3 with mannan led to synergistic increase but with curdlan resulted in inhibition (Supplementary table 1). There were no significant differences between *S.haematobium*-infected and uninfected schoolchildren in these interactions. No differences in unstimulated responses were observed between infection groups or between pre- and post-treatment responses (Supplementary figure 1A).

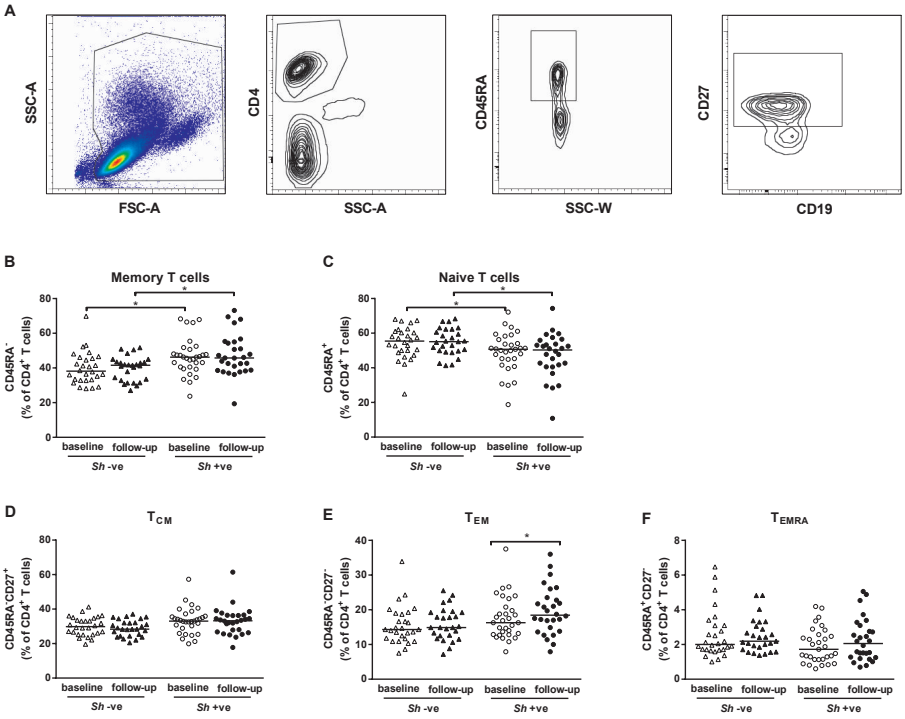


Figure 3: Memory T cell analysis at baseline and 7 months follow-up.
$CD4^+$ T cells were identified and Boolean gating combinations were used to determine proportions of T cell subsets based on CD45RA and CD27 expression (A). Proportion of $CD4^+$-gated cells that were $CD45RA^-$ (B, memory), $CD45RA^+$ (C, naïve), $CD45RA^-CD27^+$ (D, T_{CM} early), $CD45RA^-CD27^-$ (E, T_{EM}) and $CD45RA^+CD27^-$ (F, T_{EMRA}) were determined for *S. haematobium*-infected and uninfected controls at baseline and follow-up. (B-F) Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as unadjusted values. Horizontal bars represent median. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

T cell memory subpopulation analysis

To investigate the effect of schistosome infection on the frequencies of circulating memory T cell subsets we compared peripheral T cells from infected and uninfected children by flow cytometry (Figure 3A). There were no significant differences in total $CD4^+$ T cell levels between schistosome-infected and –uninfected children (34.9% and 37.6% respectively; p=0.508). Naïve and total memory T cells were defined using CD45RA expression. The proportion of memory T cells was significantly higher in infected children at baseline and remained elevated at follow-up; no differences were observed between pre- and post-treatment levels (Figure 3B). Concomitantly levels of naïve T cells were significantly lower in infected children (Figure 3C). Additional staining with CD27 distinguished between central and effector memory T cells resulting in three distinct populations: central memory (T_{CM}) ($CD45RA^-CD27^+$), effector memory (T_{EM}) ($CD45RA^-CD27^-$) and terminally differentiated effector memory cells (T_{EMRA})($CD45RA^+CD27^-$)

[21]. While no differences in the proportion of various subsets were seen at baseline (Figure 3D-F), there was a significant increase in the frequency of effector memory cells (Figure 3E) in the infected children following treatment but not in the uninfected controls.

CD4⁺CD25⁺FOXP3⁺ T cells analysis

We next assessed frequencies of circulating CD4⁺CD25⁺FOXP3⁺ T cells in a subset of infected and uninfected children, at pre- and 7 months post-treatment and also included samples that were available at an additional time point of 6 weeks post-treatment. The subset of children included in this analysis was representative of the whole study population, as no differences

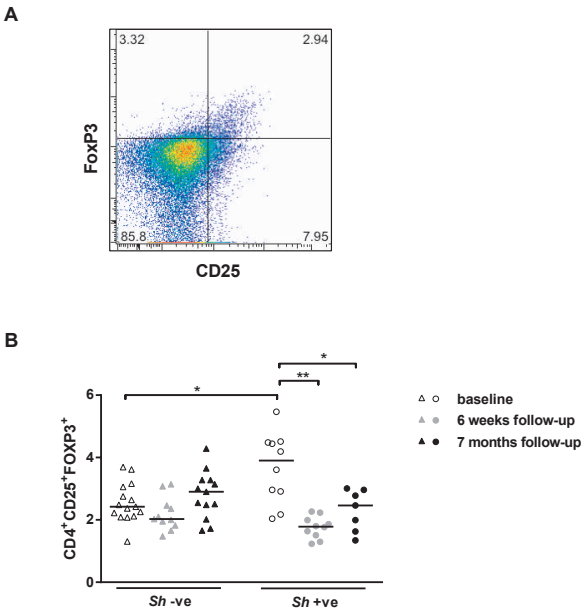


Figure 4: CD4⁺CD25⁺FOXP3⁺ T cell analysis at baseline and 6 weeks and 7 months follow-up.
CD4⁺CD25⁺FoxP3⁺ T cells were identified and enumerated with flow cytometry with initial gating steps performed like in Figure 3 (A) and frequencies determined for *S. haematobium*-infected children and uninfected controls at baseline and at follow-up (B). Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as unadjusted values. Horizontal bars represent median. * p< 0.05, ** p< 0.01, *** p< 0.001.

were found in the characteristics of this subset and the whole population. By analyzing the expression of CD4, CD25 and FOXP3 by flow cytometry (Figure 4A) we showed that the proportion of CD4⁺CD25⁺FOXP3⁺ T cells was significantly higher in infected children at baseline and these frequencies were significantly reduced to levels comparable to the uninfected control group following treatment (Figure 4B). The decrease in CD4⁺CD25⁺FOXP3⁺ T cell levels was more pronounced at 6 weeks compared to 7 months post-treatment (Figure 4B).

Association between $CD4^+CD25^+FOXP3^+$ T cells and cytokine levels in response to schistosomal products

As the decrease in $CD4^+CD25^+FOXP3^+$ T cell levels over time mirrored the increase in cytokine responses to schistosomal antigens in infected children, we further explored this longitudinal association by using linear mixed-effects models (Table 4). We assessed the relationship between $CD4^+CD25^+FOXP3^+$ T cell levels and cytokine response and found that $CD4^+CD25^+FOXP3^+$ T cell levels were significantly and negatively associated with IL-5 and IL-10 levels in response to both SEA and AWA.

Table 4: Association between $CD4^+CD25^+FOXP3^+$ T cells and cytokine levels in response to schistosomal antigens.

		S. haematobium infected children	
Cytokine	**Stimulus**	**Estimate β (95% CI)**	**p value**
IFN-γ	SEA	-0.60 (-1.61 - 0.42)	0.227
	AWA	-0.11 (-0.77 - 0.56)	0.737
IL-5	SEA	-1.04 (-1.91 - -0.18)	0.024
	AWA	-1.15 (-1.69 - -0.62)	0.001
IL-10	SEA	-1.08 (-1.76 - -0.41)	0.005
	AWA	-0.60 (-1.02 - -0.18)	0.010
TNF	SEA	-0.37 (-0.96 - 0.22)	0.188
	AWA	-0.64 (-1.43 - 0.15)	0.102
IL-2	SEA	-0.30 (-1.04 - 0.44)	0.378
	AWA	-0.47 (-0.94 - 0.00)	0.050

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; SEA, schistosomal egg antigen; AWA, adult worm antigen. Parameter estimates are from linear mixed-effects models assessing associations of level of $CD4^+CD25^+FOXP3^+$ T cells and each cytokine/stimulus combination within groups indicated by the subheadings.

Discussion

This is the first study to concurrently examine both innate and adaptive cellular immune responses, T cell memory and levels of $CD4^+CD25^+FOXP3^+$ T cells in schoolchildren infected with *S. haematobium* and to assess the effects of treatment with praziquantel on these responses. Using this approach we show that whereas antigen specific responses are suppressed during infection, this does not extend to innate immune responses. Importantly, we provide evidence that the reduction in $CD4^+CD25^+FOXP3^+$ T cells upon treatment is correlated with increase in effector T cells and antigen specific cytokine production.

In the current study whole blood innate pro-inflammatory (TNF and IL-1β) or anti-inflammatory/regulatory (IL-10 and IL-1ra) cytokine responses in response to TLR stimulation were not affected by current *S. haematobium* infection. This is in contrast to our own previous

studies which showed evidence of altered cytokine response upon TLR activation [9,12,22] and may be due to differences between using whole blood, which contain granulocytes in the current study and isolated PBMCs or cell subsets in our other studies. We also observed that IL-1β and IL-10 in response to Pam3 were lower at the 7 months follow-up time point after treatment in all subjects. This might have resulted from the change in *P. falciparum* infection and/or load which decreased in both the infected and uninfected groups over time and demonstrates why inclusion of appropriate controls, i.e. uninfected controls which are similarly followed over time is important. A prominent decrease in the levels of TNF was observed in the infected group treated with praziquantel; this is in line with the findings that show TNF to be associated with schistosome infection [23,24]. The function of DC-SIGN/MR and Dectin-1 which do not generally induce cytokine responses on their own can be studied by looking at their ability to enhance TLR responses. We did not find any differences between infected and uninfected schoolchildren or between pre- and post-treatment responses in the combined TLR and CLR stimulations.

For adaptive responses anti-schistosome treatment over a 7 month period resulted in enhanced responses to both SEA and AWA. Enhanced cytokine responses at 6-7 weeks post-treatment have been seen in a number of studies before [6–8,25]. Recently, a study examining immune responses at 1- and 2-yr after treatment of Kenyan schoolchildren infected with *S. mansoni*, observed an increase in Th2 responses (IL-4, IL-5, IL-9 and IL-13) to both SEA and AWA, however pro-inflammatory responses (IL-1β, TNF, CCL5 and IL-6) were either decreased in response to SEA or unchanged in response to AWA following treatment [26]. It is interesting to speculate that the decrease in IL-1β, TNF, CCL5 and IL-6 in the Kenyan cohort may represent an innate component of the immune response and may be in line with our own results where a decrease in TNF was observed following treatment. However, it should be noted that the children recruited in the Kenyan study all presented with hepatomegaly and exhibited predominant pro-inflammatory responses at pre-treatment whereas children in our study were free of pathology as assessed by ultrasonography of the bladder (unpublished data). Our current study indicates that the removal of parasites over a longer period of time in children without pathology is associated with sustained enhancement of antigen-specific responses, in agreement with the short-term treatment studies and with the increase in Th2 responses seen in the long-term treatment in the Kenyan study.

Down-regulation of immune responses has been ascribed to a strong immunomodulatory network induced by schistosomes [27]. In this study we show that *S. haematobium* infection is associated with increased frequency of $CD4^+CD25^+FOXP3^+$ T cells and as seen previously for $CD4^+CD25^{hi}$ Tregs in *S. mansoni* infected adults [10] $CD4^+CD25^+FOXP3^+$ T cell numbers decrease after removal of infection. Importantly, the decrease in the level of $CD4^+CD25^+FOXP3^+$ T cells over time following treatment was inversely associated with an increase in IL-5 and IL-10 cytokine production. The source of IL-10 measured here is not clear as cells other than $CD4^+CD25^+FOXP3^+$ T cells are also known to produce IL-10. Moreover this may reflect a general increase of responsiveness leading to more cytokines and possible expansion of schistosome-specific T cell responses as suggested by the increase in effector memory T cells. Future functional studies should address the mechanism by which $CD4^+CD25^+FOXP3^+$ T cells exert

their suppressive function. In addition, $CD4^+$ T cell intrinsic regulation via increased expression of surface inhibitory receptors such as CD200R [28] or increased expression of the E3 ubiquitin ligase GRAIL (gene related to anergy in lymphocytes) [29] may also play a role in observed hypo-responsiveness and likewise warrants further investigation.

When evaluating total memory T cells, we found higher proportions of memory T cells in infected children suggesting that exposure to parasite antigens drives the expansion of the memory T cell pool. This is in contrast to a recent study from Zimbabwe which showed a decrease in $CD4^+$ memory T cells, although this difference was only observed in individuals older than 13 years of age [30]. We specifically observed an increase in the effector memory T (T_{EM}) cells in the infected children following treatment which mirrored the increase in adaptive cytokine production suggesting that hypo-responsiveness may also in part be linked to the memory T cell pool. Whether this hypo-responsiveness is due to decreased cell numbers kept under control by regulatory responses or cell quiescence and/or exhaustion of effector cells, remains to be investigated. In this regard, a recent interesting study of the memory T cell pool in chronic hepatitis infection before and after treatment has shown that inhibitory molecules, PD-1 and CD244, on memory T cells were down-regulated following treatment while effector molecules perforin, granzyme B and IFN-γ were up-regulated resulting in enhanced recovery of memory T cells [31]. Future studies should investigate the phenotype of memory T cells in more depth to further understand how the phenotypic profiles affect the functional capacities of the $CD4^+$ T cells.

In summary, this study shows that infection with *S. haematobium* is associated with antigen-specific hypo-responsiveness, alterations of the T cell memory pool and increased levels of $CD4^+CD25^+FOXP3^+$ T cells and that praziquantel treatment leads to increased effector T cell frequencies and decreased levels of $CD4^+CD25^+FOXP3^+$ T cells which was associated with increased antigen-specific cytokine production.

Materials and methods

Ethics statement

The study was approved by the "Comité d'Ethique Régional Indépendent de Lambaréné" (CERIL; N°06/08). Written informed consent was obtained from parents or legal guardians of all schoolchildren participating in the study.

Study population and parasitology

Between November 2008 and February 2009, 100 schoolchildren were invited to participate in a longitudinal study to investigate the effect of *S. haematobium* infection on innate and adaptive immune responses. Children were recruited from a rural area surrounding Lambaréné (Gabon) where *S. haematobium* infection is endemic as previously described [12]. To be included in the study, participants had to meet the following criteria: (1) attendance at the local school; (2) no praziquantel treatment in the previous year; (3) no fever (body temperature < 37.5°C) and (4) no other symptoms of acute illness. Seventy-nine schoolchildren returned for follow-up performed 7 months later between June and September 2009. There were no significant

differences between the schoolchildren that returned for follow-up and those that did not.

S. haematobium infection was determined prior to blood collection by examining a filtrate of 10 mL of urine passed through a 12-µm-poresize filter (Millipore). Children were classified *S. haematobium*-infected if at least one *S. haematobium* egg was detected in the urine, or uninfected if three consecutive urine samples were negative. Infections with intestinal helminths *Ascaris lumbricoides, Trichuris trichiura* and hookworm were determined by analyzing one fresh stool sample using the Kato-Katz method [18]. Infection with *Plasmodium falciparum* was determined by PCR [19] with a cut-off value of 35 Ct. After collection of blood samples, all *S. haematobium*-infected children were treated with a single dose of praziquantel (40 mg/kg) three times every two months. Intestinal helminth- and malaria-infected children received respectively a single dose of albendazole (400 mg) or an artemisinin-based combination therapy as per the local guidelines.

Hematological parameters were analyzed using ADVIA 120 Hematology System (Bayer Health Care) and erythrocyte sedimentation rate was determined manually.

Circulating anodic antigen (CAA) testing of plasma samples was performed using the upconverting phosphor (UCP) technology lateral flow assay as previously described [20].

Reagent strips (Cobas) were used to assess hematuria, leukocyturia and proteinuria. Individual scores for each of these parameters, ranging from 0 to 3, were added together for calculation of the combined reagent strip index (RSI).

Whole blood culture

Five hours following venipuncture, heparinized blood was diluted 2 times with RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin (Astellas), 10 µg/mL streptomycin, 1 mM pyruvate and 2 mM L-glutamine (all from Sigma-Aldrich). To assess adaptive responses whole blood was incubated for 72 hours with *S. haematobium* soluble egg antigen (SEA) or adult worm antigen (AWA) (both 10 µg/mL) or 2 µg/mL PHA (Remel). To assess innate responses whole blood was incubated for 24 hours with 100 ng/mL Pam3CSK-SKKKK (EMC Microcollection), 100 µg/mL mannan (Sigma-Aldrich) or 100 µg/mL curdlan (prepared in 15mM NaOH; Wako Chemicals) alone or in combination. Medium without stimulus was used as a negative control. 100 µL of ligand(s) in medium was added to wells containing 100 µL of diluted blood in 96-well round bottom plates (Nunc) and incubated at 37°C in the presence of 5% CO_2. Supernatants were stored at -80°C.

Cytokine analysis

Supernatants were analyzed simultaneously for IFN-γ, IL-2, IL-5, IL-10 and TNF or IL-10, IL-1β, IL-1ra and TNF using customized Luminex cytokine kits (Invitrogen) according to manufacturers' recommendations. Samples were acquired with Luminex-100 cytometer (Luminex Corporation). Samples with concentrations below the detection limit were assigned values corresponding to half of the lowest value detected. For the IFN-γ, IL-2, IL-5, IL-10 and TNF multiplex assay the lowest values detected were 0.090 pg/mL for IFN-γ, 0.063 pg/mL for IL-2, 0.044 pg/mL for IL-5, 0.063 pg/mL for IL-10, and 0.051 pg/mL for TNF. For the IL-10, IL-1β, IL-1ra and TNF multiplex assay the lowest values detected were 0.016 pg/mL for IL-10, 0.255 pg/mL for IL-1β, 2.927 pg/

mL for IL-1RA and 0.016 pg/m for TNF.

Characterization of memory and CD4$^+$CD25$^+$FOXP3$^+$ T cells in peripheral blood

For immunophenotyping memory T cells freshly isolated PBMCs were fixed in 2.4% formaldehyde solution (Sigma-Aldrich) for 15 minutes at room temperature and, subsequently, frozen in RPMI 1640 medium supplemented with 20% fetal bovine serum (Greiner Bio-One) and 10% dimethyl sulfoxide (Merck) and stored at −80°C until flow cytometric analysis. After thawing, cells were washed and stained for 30 minutes with anti-CD4-PE-Cy7 (RPA-T4; eBioscience), anti-CD45RA-Horizon V450 (HI100; BD Biosciences) and anti-CD27-FITC (L128; BD Biosciences). To assess levels of CD4$^+$CD25$^+$FOXP3$^+$ T cells, ex-vivo PBMC were fixed with the FOXP3 fixation/permeabilization kit (eBisocience) and stored as described above. After thawing cells were stained with CD4-PE-Cy7 (SK3; BD Biosciences), CD25-PE (2A3; BD Biosciences) and FOXP3-APC (PCH101; eBioscience). Cells were acquired on FACSCanto II flow cytometer (BD Biosciences) and analyzed in FlowJo software v9 (Tree Star Inc.); Boolean gates were used for memory T cell analysis.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 20.

Differences between the study groups for gender, malaria and intestinal helminth infections were tested using Fisher's exact test. Differences in malaria prevalence pre- to post-treatment were tested with the McNemar's test. Age, erythrocyte sedimentation rate (ESR) and hemogram parameters were normally distributed and differences between study groups were tested using the independent student's T test. Infection indices, including schistosome egg counts, plasma CAA levels, hematuria and RSI scores and T cell subset differences were compared with the Mann-Whitney U test. Within group differences between baseline and follow-up were compared with the Wilcoxon matched pairs test. Correlations between infection indices were analyzed by Spearman's rank correlation.

Cytokine concentrations in response to stimulation were corrected for spontaneous cytokine production by subtracting responses of unstimulated medium wells to obtain net cytokine responses, with negatives values set to half of the lowest value detected per given cytokine. Net cytokine responses were normalized by log(base 10)-transformation after adding 1 pg/mL.

Interaction ratio between combined TLR and CLR stimulation was calculated by dividing net cytokine response to PAM3 in combination with mannan or curdlan by the sum of net cytokine responses obtained with each ligand alone i.e. [PAM3 + mannan]/([PAM3] + [mannan]) or [PAM3 + curdlan]/([PAM3] + [curdlan]). The interaction ratio could result in a synergistic or inhibitory effect. A paired samples T test was used to test whether the resulting interaction was significant by comparing combined stimulation with the sum of cytokines produced in two separate stimulations.

Univariate GLM analysis adjusted for age was used to compare between infection groups and the resulting adjusted means were anti-log-transformed for graphing purposes. Differences within the same group between baseline and follow-up were compared by paired samples T

test.

Linear mixed-effects model was used to assess the longitudinal association between $CD4^+CD25^+FOXP3^+$ T cell levels and cytokine responses to schistosomal products with cytokine levels as the dependent variable; participant ID as a random effect; infection group as a fixed effect; and $CD4^+CD25^+FOXP3^+$ T cell level as the covariate. The variance–covariance structure "Toeplitz" was used.

For all tests, statistical significance was considered at the 5% level. To adjust for multiple comparisons, where applicable, Bonferroni-Holm correction was taken into account.

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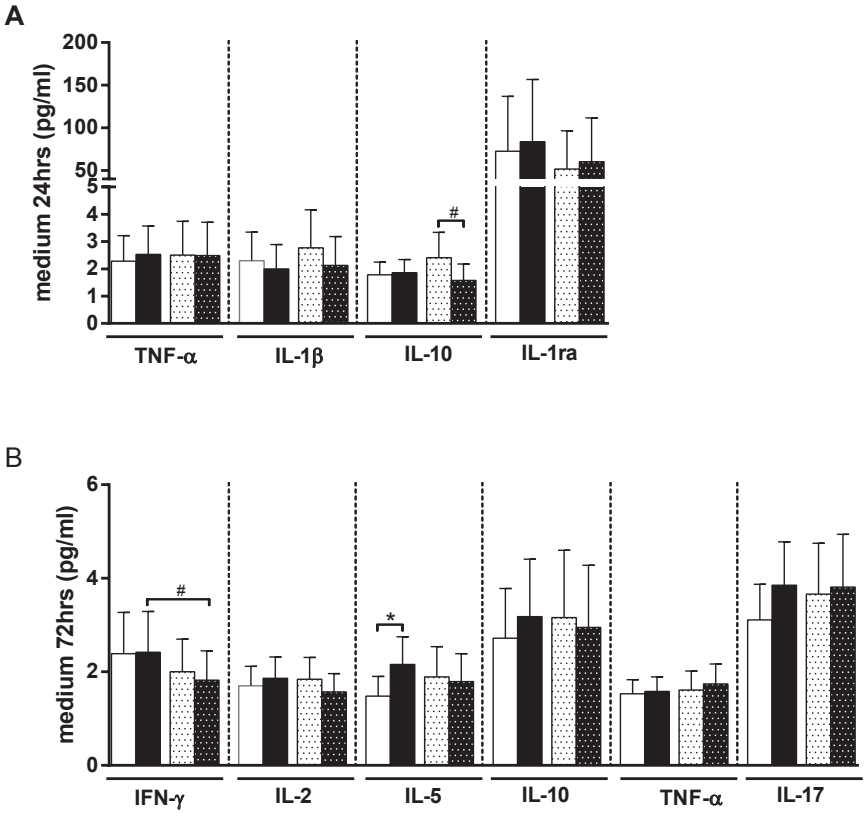
Supplementary material



Supplementary Figure 1: Cytokine production in 24hr and 72hr unstimulated whole blood cultures at baseline and 7 months follow-up.

Cytokine levels were measured in 24hr unstimulated cultures (A) by Luminex (IL-5, IFN-γ, IL-2, IL-10 and TNF). Cytokine levels were measured in 72hr unstimulated cultures (B) by Luminex (TNF, IL-1β, IL-10 and IL-1ra). Differences between groups were tested with GLM Univariate analysis adjusted for age and within groups with a paired samples T test. Results are shown as age-adjusted geometric means.

Supplementary table 1: Interaction between TLR and non-TLR ligands.

		S. haematobium uninfected		*S. haematobium* infected	
Cytokine	**Stimulus combination**	**Interaction ratio (95% CI)**	**p value**	**Interaction ratio (95% CI)**	**p value**
TNF	Pam3 + mannan	1.86 (1.19 - 2.91)	0.005	1.43 (0.90 - 2.25)	0.016
	Pam3 + curdlan	16.22 (9.89 - 26.60)	<0.001	10.76 (6.57 - 17.67)	<0.001
IL-1β	Pam3 + mannan	2.69 (2.29 - 3.15)	<0.001	2.48 (2.12 - 2.90)	<0.001
	Pam3 + curdlan	7.96 (6.26 - 10.15)	<0.001	9.79 (7.71 - 12.42)	<0.001
IL-10	Pam3 + mannan	1.29 (1.15 - 1.45)	<0.001	1.26 (1.13 - 1.42)	<0.001
	Pam3 + curdlan	0.73 (0.60 - 0.88)	<0.001	0.71 (0.59 - 0.86)	0.002
IL--1ra	Pam3 + mannan	1.15 (1.06 - 1.25)	<0.001	1.06 (.98 - 1.15)	0.225
	Pam3 + curdlan	0.59 (0.52 - 0.67)	<0.001	0.54 (0.48 - 0.61)	<0.001

TNF, tumor necrosis factor; IL, interleukin. Interaction ratio: [PAM3 + mannan]/([PAM3] + [mannan]) or [PAM3 + curdlan]/([PAM3] + [curdlan]). A paired samples T test was used to test whether the resulting interaction was significant by comparing combined stimulation with the sum of cytokines produced in two separate stimulations. All p values below 0.05 remained significant after α-adjustment by the Bonferroni-Holm method.

5

Pronounced CD4^{+}CD25^{hi}FOXP3^{+} regulatory T cell activity in human schistosomiasis before and after treatment with praziquantel.

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Abstract

Human schistosomiasis, one of the most common parasitic infections worldwide, survives within its immunocompetent host for several years. This is thought to be due to its ability to down-regulate host immune responses. In this study we examined the effect of $CD4^{+}CD25^{hi}FOXP3^{+}$ regulatory T cells (Tregs) on cell proliferation and cytokine production in response to schistosome egg (SEA) and adult worm (AWA) antigens and to the vaccine-antigen Bacillus Calmette–Guérin (BCG). These responses were studied in peripheral blood mononuclear cells (PBMC) of children infected with *Schistosoma haematobium* before and 6 weeks after anthelmintic treatment with praziquantel. Schistosome infection was associated with increased frequencies of Treg cells, which decreased following treatment. The measured cytokine (IFN-γ, IL-5, IL-10, IL-13, IL-17 and TNF) data was integrated using Principal Component Analysis (PCA). The cytokines clustered into two principal components, with principal component 1 (PC1) reflecting regulatory and Th2-polarized cytokine responses and principal component 2 (PC2) reflecting pro-inflammatory and Th1-polarized cytokine responses. The reduction in Treg frequencies after treatment was accompanied by an increase in both PC1 and PC2 following treatment compared to pre-treatment values. Proliferation, measured by CFSE dilution of dividing cells, showed no significant difference from pre- to post-treatment. To specifically assess Treg function, PBMC responses to antigens was measured after Treg depletion. Treg depletion resulted in increased values of both PC1 and PC2 at both pre-treatment and also at 6 weeks post-treatment. Proliferative responses were for the most part only significantly affected by Treg depletion in infected individuals at pre-treatment. These results show that $CD4^{+}CD25^{+}FOXP3^{+}$ T cells are higher in *S. haematobium*-infected subjects and decrease after treatment. Functionally, their suppressive activity on cytokine production does not change after treatment but their ability to influence proliferation weakens with treatment.

Introduction

The immune system has evolved several regulatory mechanisms to maintain immune homeostasis, prevent autoimmunity and restrain inflammation [1–3]. Many pathogens have developed mechanisms to manipulate the regulatory network of the host to their advantage, thereby generating conditions that ensure their survival for a prolonged period of time. In particular FOXP3$^+$ regulatory T cells (Tregs) have been shown to play a major role in the control of various parasitic infections suppressing local tissue damage and pathology that would result from otherwise over-reactivity. However, enhanced Treg cell activity may also allow the long-term survival of the parasite as the host is hampered from fighting the intruding pathogen effectively [4].

Schistosomiasis is a helminth infection affecting over 240 million people worldwide, especially children [5]. When chronic in nature it has been shown to be associated with general T cell hypo-responsiveness – evident from down-modulated antigen-specific Th1 and Th2 cell responses [6,7]. This might results from mechanisms involving peripheral anergy and suppression triggered by regulatory cells, such as Tregs [8]. For example, in experimental murine models, it was observed that the presence of Tregs suppressed the development of pathology [9], and down-modulated adequate Th1 and Th2 responses [10,11], promoting parasite survival within the host [12,13]. Evidence for Treg cell activity in human helminth infections has been provided by the detection of T cells with a regulatory phenotype in lymphatic filariasis- [14], in onchocerciasis- [15,16] and in schistosomiasis patients [17].

The killing of the schistosome parasites using effective chemotherapy with praziquantel (PZQ) has previously been shown to result in elevated Ag-specific proliferation and cytokine production, in particular IL-4, IL-5, and IFN-γ [6,18–21]. Although, the frequency of Treg cells, defined phenotypically as CD4$^+$CD25^{hi}FOXP3$^+$, appears to decrease substantially after treatment with praziquantel [17], their functional activity has not been studied before. One previous study established a positive correlation between Treg proportion and infection intensity only in children up to the age of 14, while in children > 14 years of age the opposite was the case [22].

Few studies have investigated the frequency and functional capacity of regulatory T cells on responses to helminth and vaccine antigens in infected children not only before but also after anthelmintic treatment. In the current longitudinal study we enumerated regulatory T cells in peripheral blood of *S. haematobium*-infected children at baseline and 6 weeks after treatment. We also evaluated proliferative and cytokine responses in total and Treg-depleted PBMC, both at pre- and post-treatment in order to determine the functional activity and capacity of Tregs in presence and absence of infection.

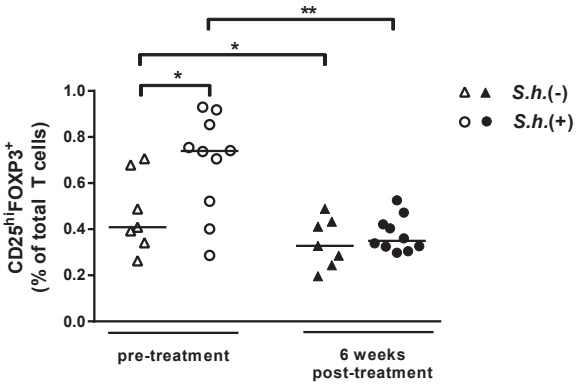
Results

Elevated levels of CD4$^+$CD25^{hi}FOXP3$^+$ Treg cells in *S. haematobium*-infected schoolchildren

To investigate whether *Schistosoma haematobium* infection affects the frequency of peripheral blood Treg cells we compared circulating CD4$^+$CD25^{hi}FOXP3$^+$ Treg cells from infected and

Figure 1: Increased frequency of CD25^{hi}FOXP3^{+} Treg cells during *S. haematobium* infection.

CD4 T cells were identified and Boolean gating combinations were used to determine proportions of CD4^{+}CD25^{hi}FOXP3^{+} Treg cells. Differences between groups were tested with a Mann-Whitney U test and within groups with a Wilcoxon matched pairs test. Horizontal bars represent median. * p< 0.05, ** p< 0.01.



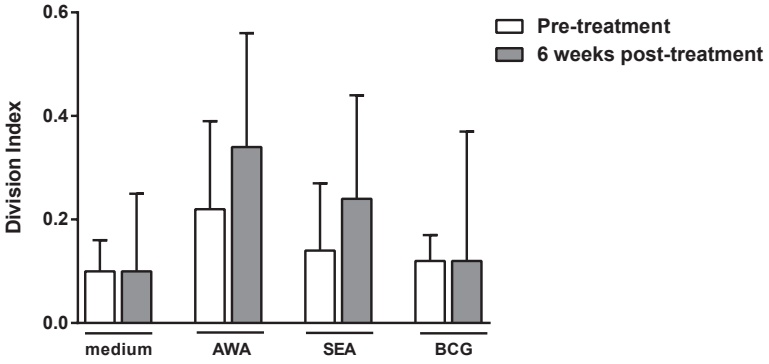


Figure 2: Proliferative responses to schistosome specific and non-specific antigens.

CFSE-labeled PBMC pre- and 6 weeks post-treatment were left unstimulated (medium), or stimulated with *S. haematobium* adult worm antigen (AWA) and soluble egg antigen (SEA) and Bacillus Calmette–Guérin (BCG). After 4 days of culture cells were fixed, cryopreserved and after thawing CFSE division was analyzed by flow cytometry. Results are shown as median with IQR. Differences between pre-treatment and 6 weeks post-treatment responses were tested with a Wilcoxon matched pairs test.

Table 1: Description of Principal Components.

	Principal Component	
	1	**2**
IL-5	**0.897**	-0.250
IL-10	**0.774**	0.076
IL-13	**0.926**	-0.129
IL-17	0.080	**0.664**
IFN-γ	-0.351	**0.853**
TNF	-0.098	**0.875**

Arbitrary values indicate the relative loading of each cytokine response towards each principal component. Strong positive loadings (>0.500) are indicated in bold. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

uninfected children by flow cytometry. We found that frequencies of $CD4^{+}CD25^{hi}FOXP3^{+}$ Treg cells were significantly higher in infected children compared to uninfected children (Figure 1). Importantly, 6 weeks after praziquantel treatment Treg frequencies were significantly reduced to levels comparable to the uninfected control group. Treg frequencies in the uninfected group were also reduced over the same period although much less so.

Proliferation and cytokine production in response to schistosome-specific and non-specific antigens in *S. haematobium*-infected schoolchildren at pre-treatment and 6 weeks post-treatment

Next, we assessed the effect of anthelmintic treatment on cell proliferation and cytokine production in response to stimulation with schistosome-specific antigens SEA and AWA and a non-specific antigen BCG; raw cytokine values are shown in supplementary table 2. Proliferation was determined by calculating the division index on the basis of the dilution of CFSE in PBMCs. There were no significant differences in proliferation between pre-treatment and 6 weeks post-treatment responses (Figure 2). Cytokine production on the other hand significantly changed between pre-treatment and 6 weeks post-treatment. We applied Principle Component Analysis (PCA) in order to provide a more global assessment of the effect of schistosome infection on responses to not only SEA and AWA stimulation but also to a third-party antigen BCG. PCA converts a large dataset of variables into a set of summary variables called principal components which represent variables that share a high level of correlation. Two distinct principal components were identified: principle component 1 (PC1) which reflects regulatory and Th2-polarized cytokine responses due to its positive loading with IL-5, IL-10 and IL-13 responses; and principle component 2 (PC2) which reflects pro-inflammatory and Th1-polarized cytokine responses due to its positive loading with IFN-γ, IL-17 and TNF (Figure 3, Table 1 and Table 2). We saw a significant increase in both PC1 and PC2 following treatment compared to baseline values (Table 3).

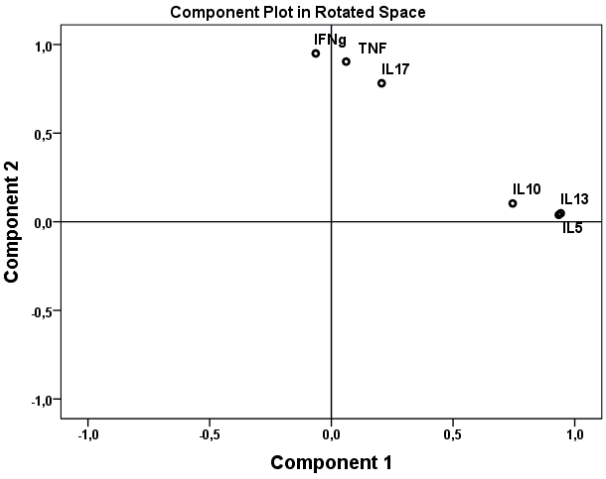


Figure 3: Principal component analysis (PCA) of cytokine responses to schistosome specific and non-specific antigens.
Two distinct principal components were identified: principle component 1 (PC1) which reflects regulatory and Th2-polarized cytokine responses due to its positive loading with IL-5, IL-10 and IL-13 responses; and principle component 2 (PC2) which reflects pro-inflammatory and Th1-polarized cytokine responses due to its positive loading with IFN-γ, IL-17 and TNF.

Table 2: Variance explained by the principal components extracted from the cytokine data.

	Initial Eigenvalues		
Component	**Total**	**% of variance**	**Cumulative %**
1	2.86	40.01	40.01
2	1.56	33.65	73.66

Two principal components were extracted from the data. The percentage of the data explained by each of the components individually and cumulatively is shown.

Table 3: Changes in cytokine production in response to antigens pre- to post-treatment.

Component	Time-point	Mean Rank	p value
1	pre-treatment	27.04	0.004
	post-treatment	41.14	
2	pre-treatment	31.41	0.021
	post-treatment	38.07	

Enhanced T cell proliferation in PBMC from schistosome-infected children after Treg depletion

To study the suppressive effect of Treg cells on proliferation and cytokine responses, $CD4^+CD25^{hi}FOXP3^+$ T cells were depleted from PBMC by magnetic beads. The $CD4^+CD25^{hi}FOXP3^+$ population decreased by 60%, $p < 0.0001$; a representative example is shown in Figure 4.

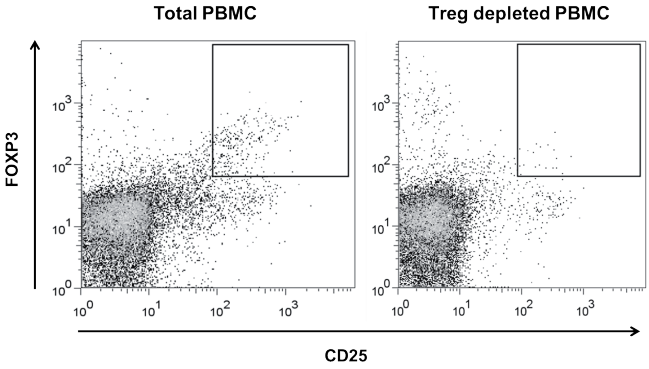


Figure 4: Treg depletion. $CD4^+CD25^{hi}$ T cells were depleted by magnetic bead separation. Representative examples of the depletion of the $CD4^+CD25^{hi}FOXP3^+$ population.

Depletion of Treg cells at pre-treatment resulted in enhanced spontaneous proliferation (medium condition) as well as in enhanced proliferation to specific schistosomal antigens AWA and SEA and to vaccine antigen BCG (Figure 5A). Interestingly, at 6 weeks after anthelmintic treatment Treg depletion resulted in significant increase in proliferation in response to AWA only. A typical plot of CFSE staining showing the effect induced by depletion of Treg cells (Figure 5B).

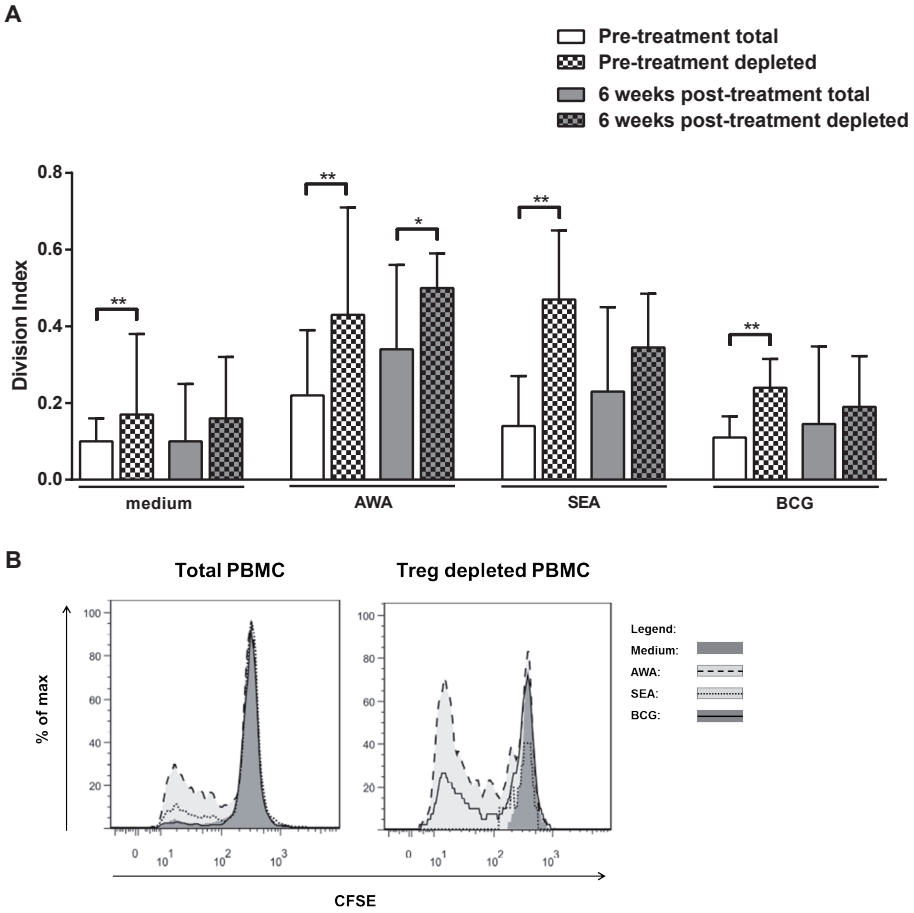


Figure 5: Effect of Treg depletion on proliferative responses to schistosome specific and non-specific antigens.

CFSE-labeled total or $CD4^+CD25^{hi}FOXP3^+$ depleted PBMC pre- and 6 weeks post-treatment were left unstimulated (medium), or stimulated with *S. haematobium* adult worm antigen (AWA) and soluble egg antigen (SEA) and Bacillus Calmette–Guérin (BCG). After 4 days of culture cells were fixed, cryopreserved and after thawing CFSE division was analyzed by flow cytometry. Results are shown as median with IQR (A). Differences between total and depleted PBMC were tested with a Wilcoxon matched pairs test. * $p< 0.05$, ** $p< 0.01$. Representative plot of CFSE staining illustrating proliferation of total or Treg depleted PBMC (B).

Increased cytokine responses following Treg depletion

Next, we investigated the capacity of Tregs to suppress cytokine responses by evaluating the effect of $CD4^+CD25^+FOXP3^+$ T cell depletion on principal component 1 (IL-5, IL-10 and IL-13) and principal component 2 (IFN-γ, IL-17 and TNF). We found $CD4^+CD25^+FOXP3^+$ T cell depletion at pre-treatment resulted in increased values of both PC1 and PC2 in infected individuals, and similarly following treatment we also observed an increase in the values of both PC1 and PC2 in the now infection free schoolchildren (Table 4).

Table 5: Changes in cytokine production in response to antigens in total and Treg depleted PBMC at pre- and post-treatment.

Component	Time-point	Total/ Treg depleted PBMCs	Mean Rank	p value
1	pre-tx	total	32.78	0.002
		depleted	42.36	
	post-tx	total	33.58	0.002
		depleted	39.38	
2	pre-tx	total	28.09	<0.001
		depleted	43.36	
	post-tx	total	32.35	<0.001
		depleted	39.41	

tx, treatment.

Discussion

Down-regulation of immune responses has been attributed to a strong immunomodulatory network induced by schistosomes and is mediated most prominently by regulatory T cells [23]. Here we demonstrate that $CD4^+CD25^+FOXP3^+$ Treg cells are increased during *S. haematobium* infection and their numbers decrease after removal of infection with praziquantel. A much smaller, yet statistically significant decrease was also observed in the frequency of these cells in the control uninfected group. This change shows the importance of including controls that could indicate any technical or environmental changes that might be associated with longitudinal studies.

In order to obtain a global assessment of the effect of *S. haematobium* infection on Th1, Th2, regulatory and pro-inflammatory cytokine responses we applied PCA analysis. This allowed us to summarize the various responses into two principal components [24]. Principle component 1 (PC1) reflected regulatory and Th2-polarized cytokine responses due to its positive loading with IL-5, IL-10 and IL-13, responses commonly associated with chronic schistosome infections. Principle component 2 (PC2) reflected pro-inflammatory and Th1-polarized cytokine responses due to its positive loading with IFN-γ, IL-17 and TNF, responses more commonly associated with acute schistosome infection or bacterial infections such as tuberculosis. We show that *S. haematobium* infection is associated with hypo-responsiveness as demonstrated by increases in cytokine production represented by both PC1 and PC2. T cell division was also assessed, but despite the consistently higher proliferation to all stimuli tested, at post treatment, the change was not statistically significant. These data indicate that the increased frequency of $CD4^+CD25^+FOXP3^+$ Tregs during schistosome infection is associated with poor cytokine responsiveness.

To assess the functional capacity of the regulatory T cells, a field applicable method was used which consists of the depletion of regulatory T cells from PBMC to assess their effect on cytokine production or proliferation. The data show that depletion of Tregs is associated with increased cytokine production, of both PC1 and PC2 which means that both Th2/regulatory

and Th1/pro-inflammatory cytokine production improves. This is the case at both pre-treatment and post-treatment time points, although the increase appears to be stronger at pre-treatment. Altogether, this would suggest that even though regulatory T cell numbers change with infection, their functional capacity to induce suppression is not affected as profoundly.

We furthermore evaluated the effect of Treg depletion on cell proliferation and in contrast to cytokine responses which were equally affected at pre-and post-treatment, proliferative responses were for the most part only significantly affected by Treg depletion in infected individuals at pre-treatment. These data could be explained if the ability of Treg to suppress proliferation would need less profound or distinct suppressive mechanisms than what is required for inhibiting cytokine production. The removal of *S. haematobium* infection could then affect the Treg function partially. Tregs are thought to exert their function via a number of different mechanism including IL-10 and/or TGF-β production, IL-2 consumption, or cell-cell contact where inhibitory molecules such as CTLA-4 and PD-1 are key [25,26]. Future studies are needed to further delineate how Treg cells exert their suppressive role during the course of schistosome infection and furthermore the difference in mechanism between suppression of proliferation versus effector cytokine production. Additional alternative mechanism, such as T cell anergy due to increased expression of the E3 ubiquitin ligase GRAIL (gene related to anergy in lymphocytes) which has been shown in a mouse model to be linked to Th2 cell hypo-responsiveness should also be investigate [27].

Alternatively, recently described regulatory $CD8^+$ T cells which likewise produce IL-10, may also in part contribute to the differences observed [28,29]. $CD25^+$ cell depletion will in addition to depleting $CD4^+CD25^+FOXP3^+$ T cells also deplete the $CD8^+CD25^+FOXP3^+$ T cell population and therefore future studies are needed to re-assess the relative contributions of these different subsets. Moreover studies with more extensive panels of markers associated with suppressive T cell functions are necessary as FOXP3 expression has been shown to be transiently up-regulated on activated $CD4^+$ T cells,

In summary, this study shows that infection with *S. haematobium* is associated with alterations of the frequency and activity of $CD4^+CD25^{hi}FOXP3^+$ regulatory T cells and that these in turn affect proliferation and global cytokine responses. These data indicate that functional activity of regulatory T cells needs to be taken into consideration when studies consider coinfections, treatment or vaccine responses in areas where helminths are prevalent.

Materials and methods

Study population

Study participants were schoolchildren attending the school of PK15, approximately 15 km south of Lambaréné in the province of Moyen-Ogouée, Gabon. The area is known to be endemic for *Schistosoma haematobium* (Table 5) (28),(33). The ethical approval for the study was obtained from the Comité d' Ethique Régional Indépendant de Lambaréné (CERIL). Prior to inclusion of study participants, signed consent was obtained from parents or legal guardians. The study included 28 schoolchildren with at least one *S. haematobium* egg in the urine sample when enrolled. Exclusion criteria were (1.) anthelmintic treatment within the last 6 weeks prior

Table 5: Characteristics of the study population.

	Pre-treatment	6 weeks post-treatment
Participants (n)	28	28
Mean age in years (SD)	10.32 (2.2)	n/a
Sex (female/male)	15/13	n/a
Egg count/10 ml urine: median (IQR)	72.5 (24.5 – 296.3)	0.0 (0.0 – 1.5)
Level of haemoglobin (g/dL): mean (SD)	11.13 (0.97)	11.09 (0.94)
Level of white blood cell (x103/µl): mean (SD)	8.76 (3.18)	9.44 (2.92)

to the study, (2.) the presence of fever (tympanic body temperature > 37.5ºC) and/or (3.) others symptoms of acute illness. To compare the frequency of $CD25^{hi}FOXP3^{+}$ Treg cells between *S. haematobium* infected and uninfected schoolchildren an additional 17 participants were recruited (Supplementary table 1); 10 *S. haematobium* infected participants were recruited from PK15 and 7 uninfected participants were recruited from Lambaréné.

Detection of *S. haematobium* infection

A midstream urine sample was collected between 10:00 and 14:00 hours to coincide with the peak of *S. haematobium* egg excretion in urine (34). Infection was determined by passing 10 ml of urine through a 12.0 µm polyamide N filter (Millipore) for the detection of *S. haematobium* eggs (14).

Praziquantel treatment

S. haematobium infected schoolchildren were treated with a single dose of PZQ (40 mg/kg). Three weeks later, in order to ensure clearance of parasites, the same treatment was administered a 2nd time. Six weeks after the first treatment efficacy of PZQ was assessed by measuring the egg load in urine. Donors remaining egg-positive after the 2nd treatment were given a third dose of PZQ and excluded from analysis, if their reduction in egg count was less than 90% (N=5).

Cell isolation and depletion

Peripheral blood mononuclear cells (PBMCs) were purified from heparinized venous blood (7-10ml) by Ficoll-Hypaque centrifugation (Amersham Biosciences, Netherlands). Depletion of $CD25^{hi}$ T cells was performed using a suboptimal concentration of CD25 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

PBMC culture for proliferation and cytokine production

To analyse proliferation green-fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich, CA, USA) was used; CFSE divides over daughter cells upon cell division and can be tracked by decreasing fluorescence intensity. $CD25^{hi}$ depleted and total PBMC were stained with 2 µM CFSE for 15 minutes at room temperature prior to culture. After labelling, cells were cultured in RPMI 1640 (Gibco, Invitrogen®, Carlsbad, CA, USA), supplemented with 10% FCS

(Greiner Bio-One GmbH, Frickenhausen, Germany), 100 U/ml penicillin (Astellas, Tokyo, Japan), 10 µg/ml streptomycin, 1mM pyruvate and 2mM L-glutamine (all from Sigma-Aldrich, CA, USA). Cells were stimulated in round-bottom plates with medium, 10 µg/ml AWA, 10 µg/ml SEA or 10 µg/ml BCG and incubated in the presence of 5% CO_2 at 37.5°C. After 4 days, supernatants were collected and stored at -80°C, while cells were harvested, fixed with 2% formaldehyde (Sigma-Aldrich, CA, USA) and, subsequently, frozen in RPMI 1640 medium supplemented with 20% FCS and 10% DMSO (Merck KGaA, Darmstadt, Germany) and stored at -80°C.

Flow cytometry analysis (FACS)

After thawing, CFSE-labelled cells were incubated with CD4-PE and CD25-APC (all from BD Bioscience, San Diego, CA, USA), acquired on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) and data were analysed in a FlowJo Proliferation application (Tree Star Inc., Ashland, OR, US) by calculation of the fraction of cells that had divided from the starting population (division index). To assess levels of $CD25^{hi}FOXP3^{+}$ Treg cells, ex-vivo PBMC were fixed with the FoxP3 fixation/permeabilization kit (eBisocience, San Diego, CA, USA) and frozen in RPMI 1640 medium supplemented with 20% FBS and 10% DMSO and stored at -80°C. For immunophenotyping isolated PBMCs were stained with CD4-PE/Cy7 (SK3; BD Biosciences, San Diego, CA, USA), CD25-PE (2A3; BD Biosciences, San Diego, CA, USA) and FOXP3-APC (PCH101; eBioscience, San Diego, CA, USA), cells were acquired on FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA) and analysed in FlowJo software (Tree Star Inc., Ashland, OR, US) using Boolean combination gates.

Cytokines assays

Cytokines were measured from supernatants using Luminex 100 IS System (Invitrogen, Carlsbad, CA, USA) and commercially available beads and standards from BioSource (Bleiswijk, Netherlands) for interferon-gamma (IFN-γ), interleukin-5 (IL-5), IL-10, IL-13 and IL-17 and tumor necrosis factor (TNF). Beads were titrated for optimal dilution and used according to manufacturer's instructions.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 20.

Cytokine concentrations in response to stimulation were corrected for spontaneous cytokine production by subtracting responses of unstimulated medium wells to obtain net cytokine responses, with negatives values set to half of the lowest value detected per given cytokine.

To avoid type I and type II errors in multiple testing, immunological parameters were reduced by principal-components analysis (PCA). First, R v2.15.1 Development Core Team software (R Foundation for Statistical Computing, Vienna, Austria, 2012, http://www.R-project.org) was used to estimate Box-Cox transformation parameter for each cytokine to increase normality of the data. Principal Component Analysis with Varimax rotation was used to reduce the data into a smaller number of uncorrelated variables. Rotation converged in 3 iterations. Principal components (PC) with eigenvalues greater than 1 were selected, and PC scores were

computed for each combination of conditions: stimuli AWA/SEA/BCG; total and Treg depleted PBMC; pre- and post-treatment.

Differences between pre- and post-treatment and Treg depleted and total PBMC were tested with the Wilcoxon matched pairs test. For all tests, statistical significance was considered at the 5% level.

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Supplementary material

Supplementary table 1: Characteristics of the study population for assessment of $CD25^{hi}FOXP3^{+}$ Treg cells.

	***S.haematobium* uninfected**	***S.haematobium* infected**
Participants (n)	7	10
Mean age in years (SD)	12.9 (2.6)	12.5 (1.5)
Sex (female/male)	4/3	9/1
Egg count/10 ml urine: median (IQR)	0	19.5 (3.25 – 216.5)
Level of haemoglobin (g/dL): mean (SD)	10.86 (0.72)	11.4 (0.31)
Level of white blood cell ($x10^3$/µl): mean (SD)	6.33 (0.73)	8.82 (0.55)

Supplementary table 2: Raw cytokine values following antigen stimulation in total and Treg depleted PBMC at pre- and post-treatment.
IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; AWA, adult worm antigen; SEA, soluble egg antigen; BCG, Bacillus Calmette–Guérin (BCG)

		Pre-treatment		6 weeks post-treatment	
Cytokine	**Stimulus**	**Total (median (IQR))**	**Treg depleted (median (IQR))**	**Total (median (IQR))**	**Treg depleted (median (IQR))**
IL-5	medium	28.97 (10.61 - 91.81)	29.67 (9.75 - 90.39)	69.40 (39.08 - 154.05)	43.31 (16.58 - 149.90)
	AWA	475.86 (173.95 - 774.07)	372.44 (166.76 - 827.32)	721.88 (373.64 - 1185.76)	745.63 (337.48 - 1112.00)
	SEA	127.50 (62.61 - 241.38)	156.14 (115.71 - 615.18)	184.83 (89.09 - 469.41)	257.78 (102.46 - 440.22)
	BCG	4.96 (3.00 - 13.63)	4.48 (3.00 - 14.75)	14.11 (3.00 - 36.76)	12.67 (3.23 - 38.88)
IL-13	medium	101.43 (14.34 - 141.82)	88.75 (33.64 - 198.27)	142.17 (47.78 - 341.57)	163.38 (47.78 - 363.11)
	AWA	570.86 (339.74 - 1155.67)	798.86 (484.03 - 1237.20)	1047.52 (657.20 - 1984.41)	1359.63 (714.84 - 2126.23)
	SEA	254.60 (135.16 - 467.21)	442.24 (280.61 - 946.45)	522.26 (150.21 - 897.83)	728.83 (277.69 - 1357.21)
	BCG	32.70 (10.06 - 82.64)	85.59 (18.02 - 146.92)	83.64 (17.36 - 217.83)	98.46 (28.14 - 255.57)
IFN-γ	medium	10.01 (5.00 - 28.82)	28.82 (14.34 - 58.51)	11.37 (5.00 - 30.13)	13.36 (5.00 - 66.35)
	AWA	13.52 (5.00 - 36.19)	43.64 (12.02 - 106.81)	6.98 (5.00 - 30.10)	26.69 (5.53 - 91.46)
	SEA	8.18 (5.00 - 24.08)	30.03 (10.25 - 102.82)	6.95 (5.00 - 29.77)	41.74 (9.56 - 75.56)
	BCG	266.25 (84.88 - 911.19)	728.69 (400.98 - 2457.31)	377.74 (200.41 - 1466.34)	1262.53 (453.56 - 3996.58)
IL-10	medium	15.00 (15.00 - 28.61)	15.00 (15.00 - 17.63)	15.00 (15.00 - 42.94)	15.00 (15.00 - 32.71)
	AWA	199.99 (92.99 - 306.86)	199.40 (110.98 - 387.70)	288.56 (120.15 - 564.33)	444.80 (212.37 - 645.22)
	SEA	132.81 (65.02 - 216.76)	164.55 (84.82 - 282.93)	186.01 (77.29 - 373.99)	244.98 (86.71 - 348.82)
	BCG	63.48 (15.00 - 117.97)	63.07 (15.86 - 115.49)	135.74 (62.28 - 196.93)	130.88 (51.06 - 270.97)
IL-17	medium	10.00 (10.00 - 19.15)	10.00 (10.00 - 11.35)	10.00 (10.00 - 13.63)	10.78 (10.00 - 41.59)
	AWA	10.00 (10.00 - 31.93)	25.59 (10.00 - 60.03)	18.09 (10.00 - 41.01)	27.41 (12.39 - 82.30)
	SEA	11.52 (10.00 - 38.83)	19.11 (10.00 - 44.83)	10.00 (10.00 - 37.55)	38.63 (11.51 - 64.09)
	BCG	39.70 (10.00 - 74.94)	36.89 (20.00 - 174.11)	65.54 (20.62 - 118.98)	109.85 (22.72 - 155.26)
TNF	medium	46.29 (14.33 - 125.45)	42.53 (15.66 - 178.20)	98.50 (33.23 - 233.55)	101.56 (25.05 - 256.81)
	AWA	154.54 (85.24 - 231.39)	330.86 (167.01 - 529.55)	224.74 (101.72 - 335.88)	306.40 (122.04 - 582.24)
	SEA	119.93 (30.84 - 265.32)	201.29 (44.71 - 480.41)	94.31 (42.38 - 261.80)	172.38 (66.79 - 366.74)
	BCG	537.67 (183.00 - 1268.92)	1708.70 (738.50 - 5445.60)	1024.85 (422.46 - 2013.62)	1641.89 (675.63 - 4619.88)

6

Schistosomes induce regulatory features in human and mouse CD1d^{hi} B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells.

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Abstract

Chronic helminth infections, such as schistosomes, are negatively associated with allergic disorders. Here, using B cell IL-10-deficient mice, *Schistosoma mansoni*-mediated protection against experimental ovalbumin-induced allergic airway inflammation (AAI) was shown to be specifically dependent on IL-10-producing B cells. To study the organs involved, we transferred B cells from lungs, mesenteric lymph nodes or spleen of OVA-infected mice to recipient OVA-sensitized mice, and showed that both lung and splenic B cells reduced AAI, but only splenic B cells in an IL-10-dependent manner. Although splenic B cell protection was accompanied by elevated levels of pulmonary FoxP3$^+$ regulatory T cells, in vivo ablation of FoxP3$^+$ T cells only moderately restored AAI, indicating an important role for the direct suppressory effect of regulatory B cells. Splenic marginal zone CD1d$^+$ B cells proved to be the responsible splenic B cell subset as they produced high levels of IL-10 and induced FoxP3$^+$ T cells in vitro. Indeed, transfer of CD1d$^+$ MZ-depleted splenic B cells from infected mice restored AAI. Markedly, we found a similarly elevated population of CD1d^{hi} B cells in peripheral blood of *Schistosoma haematobium*-infected Gabonese children compared to uninfected children and these cells produced elevated levels of IL-10. Importantly, the number of IL-10-producing CD1d^{hi} B cells was reduced after anti-schistosome treatment. This study points out that in both mice and men schistosomes have the capacity to drive the development of IL-10-producing regulatory CD1d^{hi} B cells and furthermore, these are instrumental in reducing experimental allergic inflammation in mice.

Introduction

The prevalence and severity of allergic diseases and asthma has increased over the last five decades in industrialized countries [1]. Conversely, lower prevalence of allergic diseases is seen in low income countries. Many epidemiological studies have reported an inverse association between helminth infections, which are highly prevalent in developing countries, and allergic or auto-immune disorders [2–4]. In order to study the interaction between helminth infections and protection against allergic diseases, murine models of allergic airway inflammation (AAI) and helminth infection have been developed. For example, *H. polygyrus* or *T. spiralis* infections protected against house dust mite-induced and/or ovalbumin (OVA)-specific AAI [5,6]. In addition, *S. mansoni* infection protected mice against OVA-induced airway hyperresponsiveness (AHR) [7]; protection was optimal during the chronic stage of infection, but not the acute stage [8].

Helminth infections are characterized by potent type 2 effector responses and a strong regulatory network [9]. Regulatory T (Treg) cells are well-known for their suppressive capacity, but recent studies in auto-immunity models have indicated that also B cells can be important players in immune regulation [10]. These so-called regulatory B cells are known to influence the immune system by the regulatory cytokines IL-10 and TGF-β, influencing T cell proliferation, downregulating CD4^{+}, CD8^{+}, NK T cell activation and promoting FoxP3^{+} Treg cell induction [11]. Interestingly, a number of studies have reported that B cells may have an active regulatory role in various parasitic infections. For example, IL-10-producing B cells in *L. major*-infected BALB/c mice are essential in suppressing type 1 responses that are necessary to clear infection [12] and *S. mansoni*-infected B cell-deficient μMT mice show more extensive hepatic granulomas [13]. In addition, in a model of systemic anaphylaxis or AAI, in combination with *S. mansoni* infection with adult stage worms only, B cells appeared to be major players in addition to IL-10 and Treg cells [7,14].

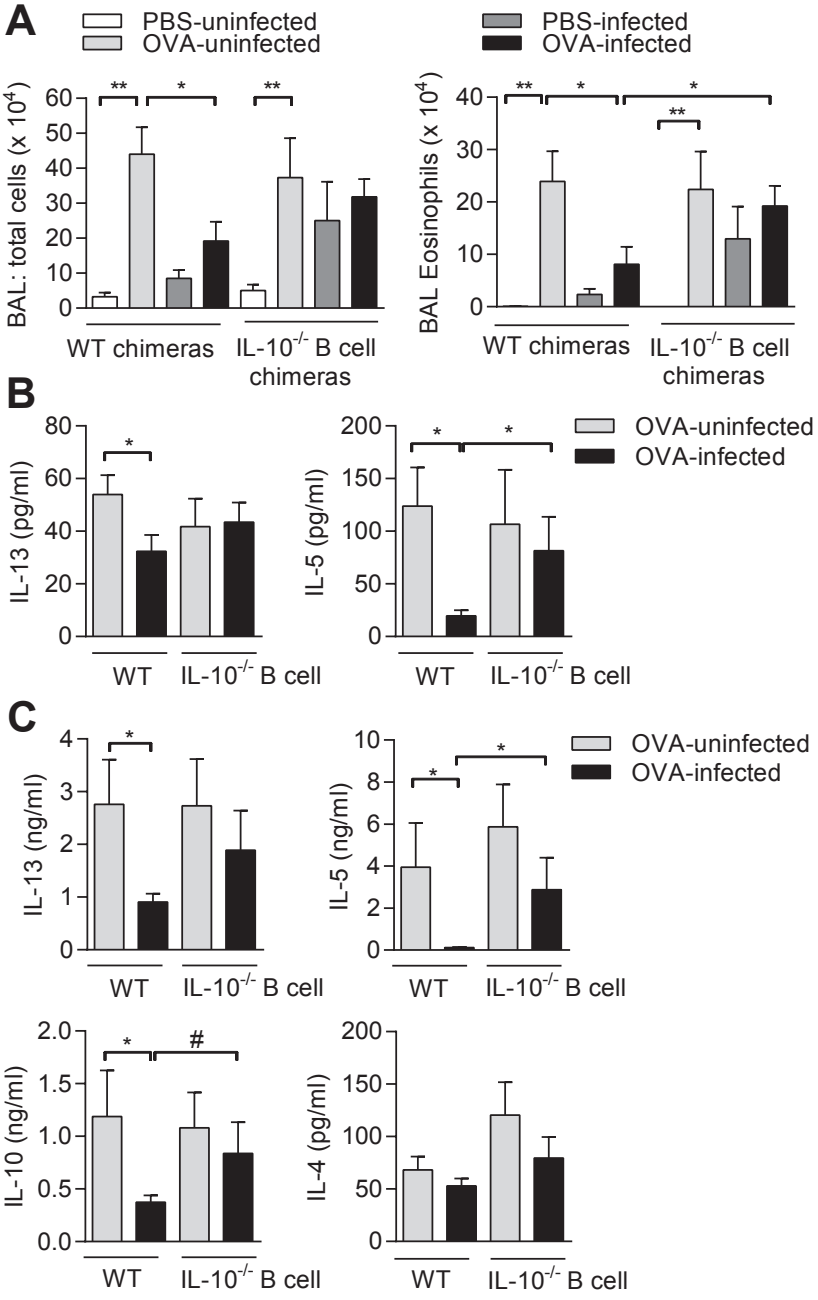
In auto-immunity models several Breg subsets have been identified, including marginal zone (MZ), transitional or CD5^{+}CD1d^{hi} B cells [15]. Recent studies in human auto-immune diseases have substantiated these findings by showing human Breg cells in peripheral blood characterized as CD24^{hi}CD38^{hi} [16], CD24^{hi}CD27^{+} [17] or CD1d^{hi} B cells [18].

Here, we investigated both in mice and humans whether schistosome infections can induce functional Breg cells. Indeed, we identified for the first time in peripheral blood of *S. haematobium*-infected children elevated numbers of IL-10-producing CD1d^{hi} regulatory B cells, which were decreased after treatment. The functional capacity of those schistosome-induced Breg cells was confirmed in a mouse model of allergic airway inflammation where Breg-derived IL-10 and Breg-induced Treg cells mediated suppression.

Results

IL-10-producing B cells are important for protection against allergic airway inflammation

Elevated IL-10 characterizes chronic stages of schistosome infection and is produced by B cells


A
PBS-uninfected
OVA-uninfected
PBS-infected
OVA-infected
BAL: total cells (x 10^4)
BAL Eosinophils (x 10^4)
WT chimeras
IL-10-/- B cell chimeras
B
IL-13 (pg/ml)
IL-5 (pg/ml)
OVA-uninfected
OVA-infected
WT
IL-10-/- B cell
C
IL-13 (ng/ml)
IL-5 (ng/ml)
IL-10 (ng/ml)
IL-4 (pg/ml)

starting from week 12 during infection (Fig. S1) [8]. To determine whether B cells are a dominant source of IL-10 and whether this IL-10 is essential for protection against AAI during chronic schistosome infections, IL-10-deficient B cell and control wild-type (WT) chimeric mice were generated and chronically infected with *S. mansoni* followed by an allergic OVA sensitization and challenge. The IL-10-deficiency was restricted to the B cells population (confirmed by intracellular flowcytometry) as described before [10]. In the uninfected allergic groups (OVA-uninfected) for both WT and IL-10$^{-/-}$ B cell chimeric mice, the bronchoalveolar lavage (BAL) fluid contained significantly more eosinophils (Fig. 1A), lymphocytes and macrophages compared to uninfected non-allergic groups (PBS-uninfected) (data not shown). Nevertheless, in OVA-infected WT mice significantly less eosinophils were found compared to OVA-uninfected WT mice (Fig. 1A), as previously described [8]. In contrast, eosinophilia in the OVA-infected group of the IL-10$^{-/-}$ B cell mice was restored and was similar to the OVA-uninfected IL-10$^{-/-}$ B cell group and significantly higher compared to the OVA-infected WT group. Interestingly, in the PBS-infected group, eosinophilia was equally high, suggesting that IL-10-producing B cells were involved in controlling non-allergic inflammatory processes during natural infections as well. Furthermore, IL-5, IL-13 and/or IL-10 were equally elevated in the BAL fluid and mediastinal lymph nodes (MedLN) of OVA-infected IL-10$^{-/-}$ B cell mice compared to OVA-uninfected IL-10$^{-/-}$ B cell group, whereas these Th2 cytokines were reduced in OVA-infected WT mice (Fig. 1B, C). The IL-5 production in the BAL fluid and by T cells in the MedLN was also significantly increased in the OVA-infected IL-10$^{-/-}$ B cells mice compared to the OVA-infected WT mice as well. As expected, IL-4 remained at low levels due to the C57/Bl6 background of the chimeric mice (Fig. 1C). These results indicate that IL-10-producing B cells are critically involved in the downmodulation of eosinophilia and the Th2 response against OVA antigen leading to protection against AAI during chronic schistosomiasis.

Pulmonary and splenic B cells produce IL-10 and protect against AAI

In order to identify the dominant organ with IL-10-producing B cells during infection, we isolated B cells from organs that have previously been described to harbor regulatory B cells (spleen), drain schistosome infection sites (mesenteric lymph nodes, MLN) or are the effector site where allergic inflammation is found (lung). Both pulmonary and splenic B cells, but not mesenteric B cells, from chronically-infected mice were able to produce IL-10 upon soluble egg antigen (SEA) stimulation, with highest production by splenic B cells (Fig. 2A). To study the suppressive activity of isolated B cells from different organs in downmodulating AAI, we adoptively transferred CD19^{+} B cells from OVA-infected mice into OVA-sensitized recipient mice. AAI was reduced by pulmonary or splenic but not by mesenteric B cells (Fig. 2B).

Figure 1. Role of IL-10-producing B cells on OVA-specific AAI during chronic *S. mansoni* infection.
Chimeric WT and IL-10$^{-/-}$ B cell mice were infected with *S. mansoni* and sensitized and challenged for OVA at week 11 and 12 after the start of infection. BAL fluid was collected and total BAL cells (A) and eosinophils were determined. (B) In the BAL fluid, IL-5 and IL-13 were measured by ELISA. (C) MedLN cells were collected and restimulated by OVA (10 µg/mL) for four days and IL-4, IL-5, IL-10, and, IL-13 production was determined using ELISA. Results are from two independent experiments and each group consists of 6 PBS- and 6 OVA-uninfected, 8 PBS- and OVA-infected mice.

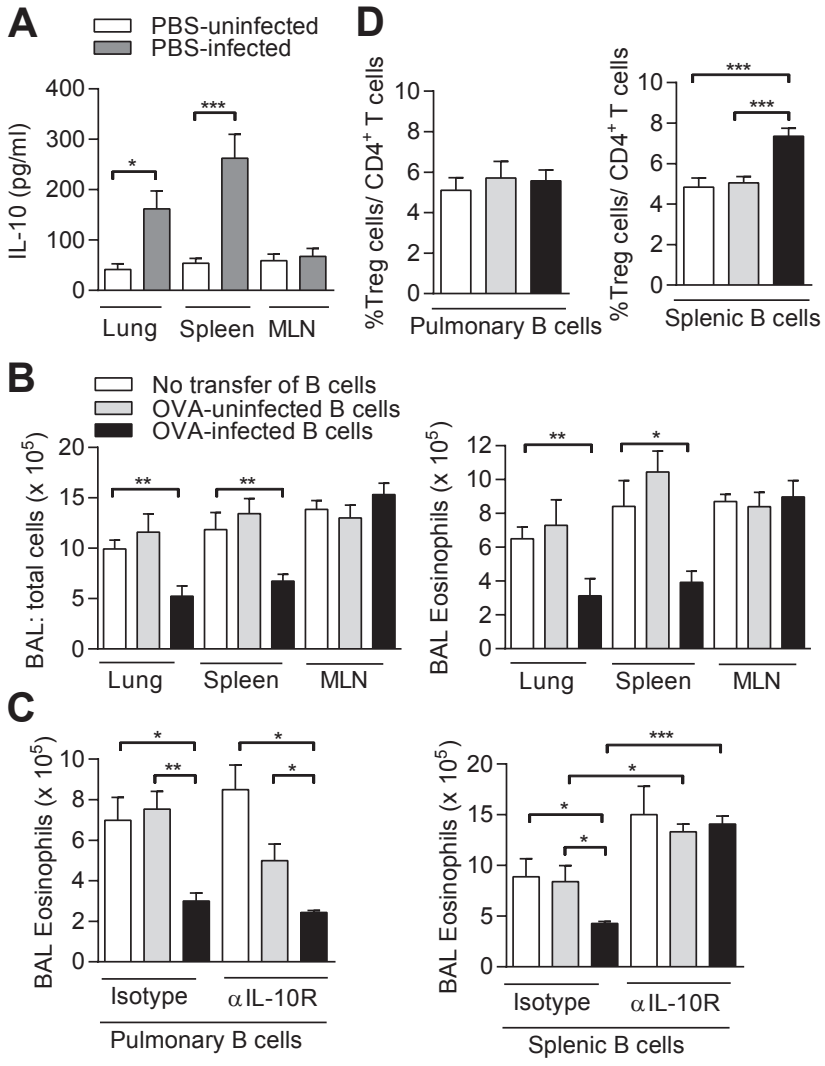


Figure 2. IL-10 production by B cells from different organs during chronic schistosomiasis and their role in protection against AAI.

(A) WT mice were treated as in Fig 1A. Splenic, pulmonary or mesenteric B cells (1 x 10^5) were isolated and cultured in the presence of SEA (20 μg/ml) for five days. IL-10 production was measured using ELISA and medium value was subtracted. (B) OVA-sensitized recipient mice received 5 x 10^6 B cells from different organs. After challenge, BAL cell numbers and eosinophils were determined (C) BAL numbers and eosinophilia of mice that received 250 μg isotype control or anti-IL-10R abs per mouse one day before the adoptive transfer. (D) The percentage of $CD4^+CD25^+FoxP3^+$ Treg cells was determined in the lungs of recipient mice. Each graph represents three independent experiments, consisting of five mice per group.

Interestingly, the protective effect of the transfer of splenic B cells, but not of pulmonary B cells, was abolished by administering a blocking IL-10 receptor antibody (Fig. 2C). Furthermore, we observed increased percentages of $CD4^+CD25^+FoxP3^+$ T cells in the lungs of recipient mice, but only after administering splenic B cells (Fig. 2D). This data indicate that pulmonary B cells can drive IL-10 and Treg cell-independent protection against eosinophilic AAI, while splenic B cells protect via an IL-10-dependent mechanism and enhance local Treg cell numbers in the lungs.

Splenic B cells induce Treg cells, which support reduction of AAI

Regulatory B cells are known to regulate inflammation by recruitment and generation of Treg

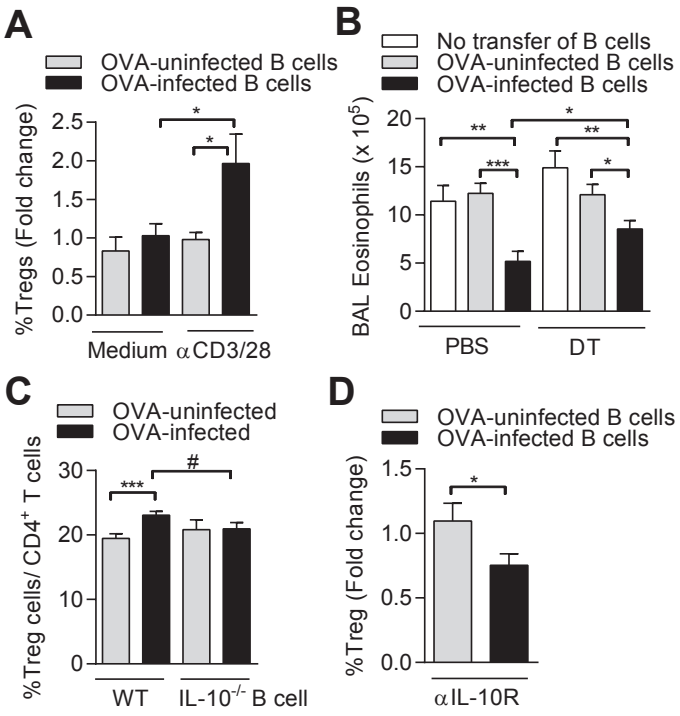


Figure 3. Treg cell induction by IL-10-producing Breg cells.
(A) Irradiated splenic B cells (1 x 10^5) were cultured with $CD4^+CD25^-$ T cells (1 x 10^5) for 5 days in the presence of anti-CD3 and anti-CD28. Induction of $CD4^+CD25^+FoxP3^+$ Treg cells (in %) by PBS-uninfected B cells was set at one. Fold change in Treg cell percentage for OVA-uninfected and OVA-infected B cells was calculated. Graph expresses results from three independent experiments. (B) OVA-sensitized DEREG mice were treated as in Fig. 2B in addition to a DT or PBS injection. This graph expresses two experiments, consisting of five mice per group. (C) WT and IL-$10^{-/-}$ B cell chimeras were treated as in Fig. 1A. The MedLNs were collected and the percentage of $CD4^+CD25^+FoxP3^+$ Treg cells was determined. Figure contains two independent experiments and each group consists of 6 to 8 mice. (D) *In vitro* co-culture were performed as described in (A) in the presence of blocking anti-IL-10R or isotype control antibodies. Percentage of Treg cells induced in the presence of isotype control was arbitrarily set at 1. Fold change in Treg cell induction in the presence of anti-IL-10R was calculated. Graph represents three independent experiments.

cells in auto-immune disorders [10]. Therefore, we cultured irradiated splenic B cells from OVA-uninfected or OVA-infected mice with $CD4^+CD25^-$ T cells from naive mice to evaluate Treg cell development. Splenic B cells from OVA-infected mice doubled the percentage of $CD4^+CD25^+FoxP3^+$Treg cells (Fig. 3A), while pulmonary B cells did not change the level of Treg cells, as already confirmed *in vivo*. In addition, no shift towards Th1 or Th2 cytokines was found during the co-culture showing that the splenic B cells primarily influenced the development of Treg cells but not of other T cell subsets (data not shown).

Next, we studied the contribution of B cell-induced Treg cells in protection against AAI in vivo using OVA-sensitized FoxP3-DTR transgenic DEREG mice[19]. The temporal loss of $FoxP3^+$ Treg cells during allergen challenge only partly restored AAI, showing that in the group with transferred B cells from OVA-infected mice, B cell-induced Treg cells are only partially involved in protection against AAI (Fig. 3B).

A causal relationship between IL-10-producing B cells and Treg cells has been suggested in

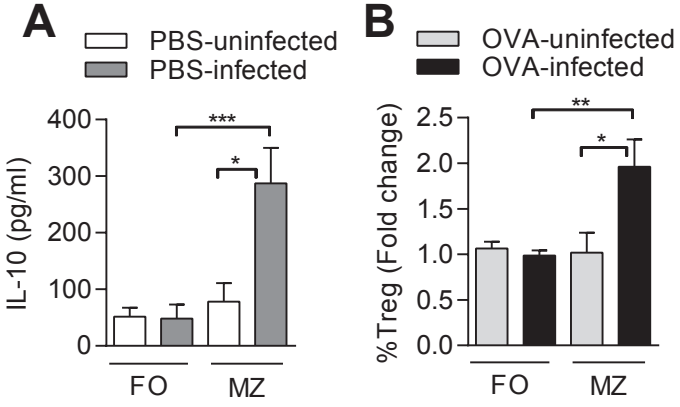


Figure 4. MZ B cells show regulatory features.
(A) MZ and FO B cells from PBS-uninfected and PBS-infected were sorted using flow cytometry and cultured for 5 days in the presence of SEA for IL-10 production as in Fig. 2A. (B) Irradiated MZ B cells or FO B cells (1 x 10^5) were co-cultured with $CD4^+CD25^-$ T cells as described in Fig. 3A. Treg cell induction by MZ or FO B cells from uninfected mice was set at one. Subsequently, fold change in Treg cell induction by MZ or FO B cells from OVA-uninfected and OVA-infected mice was calculated. Each graph contains three independent experiments with five mice per group.

antigen-induced arthritis model utilizing similar chimeric mice, where loss of IL-10-producing B cells led to significant reduction of Treg cells in draining inguinal LN[22]. In OVA-infected WT mice, we found more $CD4^+CD25^+FoxP3^+$ Treg cells in the lung-draining MedLN compared to OVA-uninfected mice, which was not found in OVA-infected IL-$10^{-/-}$ B cell mice (Fig. 3C). Using the same *in vitro* co-culture as describe above, significantly less Treg cell induction by splenic B cells was found when anti-IL-10R antibodies were added compared to isotype control (Fig. 3D), underlining the role of IL-10 for Treg cell induction both *in vivo* and *in vitro*.

Schistosome-induced MZ B cells exhibit regulatory activities

We compared the two main splenic B cell subsets, the follicular (FO) B cells and the MZ B cells (which are high in CD1d, Fig. S2) for their ability to produce IL-10 during chronic infection. Sorted subsets from uninfected and infected mice were cultured in the presence of SEA, showing that the MZ B cells from infected mice produced high IL-10 levels, while FO B cells produced only low levels (Fig. 4A). Production of IL-12 and IL-1β remained low in both subsets of infected mice, while production of TNF-α and IL-6 was increased in FO cells, but remained low in MZ B cells from infected mice (Fig. S3). Based on our findings regarding Treg cell induction by total splenic B cells, we questioned whether MZ B cells, as the strongest IL-10 producers, were also responsible for Treg cell induction in vitro. Therefore, irradiated FO and MZ B cells were cultured with CD4$^+$CD25$^-$ T cells and the highest percentage of FoxP3$^+$ T cells was observed in co-culture with MZ B cells (Fig. 4B), which was not explained by differences in proliferation as similar cell counts were found in cultures with either FO or MZ B cells (data not shown). These data indicate that MZ B cells are responsible for the regulatory features observed in total splenic B cells by

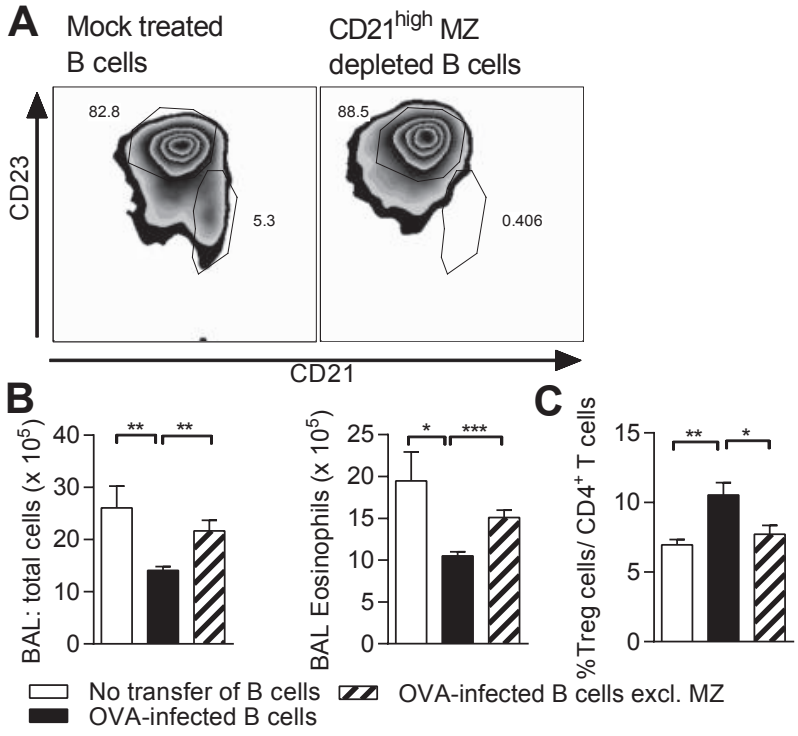


Figure 5. MZ B cells are important in the protection against AAI.
(A) Mock-depleted splenic and the MZ-depleted B cells were injected in OVA-sensitized recipient mice. After challenge, total BAL cell count and the number of BAL eosinophils (B) and the percentage of CD4$^+$CD25$^+$FoxP3$^+$ T cells in the lungs (C) was measured. Figure is representative of two independent experiments, consisting of five mice per group.

producing IL-10 and by enhancement of FoxP3$^+$ T cells.

Next, the contribution of the MZ B cells in vivo was investigated by depletion of the CD21^{hi} cells from total splenic B cells of OVA-uninfected or OVA-infected mice by indirect magnetic labeling (Fig. 5A). The transfer of mock-treated B cells from OVA-infected mice resulted in a decrease in total BAL cell count and eosinophilia (Fig. 5B), as observed before. Importantly, the transfer of CD21$^+$-depleted B cells restored the severity of AAI and the induction of pulmonary FoxP3$^+$ Treg cells was lost (Fig. 5C), confirming the significance of MZ B cells in protection against AAI in vivo.

Elevated levels of IL-10-producing CD1d^{hi} B cells in *S. haematobium*-infected Gabonese children

All data regarding schistosome-induced Breg cells stems from mouse models. Therefore, we asked whether in humans schistosomes can also induce IL-10-producing regulatory B cells. PBMC were collected from 20 Gabonese children that were either *S. haematobium* positive or negative (Table 1). All samples were simultaneously analyzed for different Breg markers,

Table 1. Demographic characteristics and infection status of Gabonese children.

	***S.h.* infected**	***S.h.* uninfected**
Participants pre-tx/post-tx	10/7	10/8
Mean age in years (range)	10.3 (8-14)	11.2 (8-14)
Sex male/female	5/5	4/6
Mean egg counts (range)	31.7 (1-201)	0
Co-infections:		
Plasmodium falciparum	4/10	1/10
Ascaris lumbricoides	3/9	3/8
Trichuris trichiura	3/9	6/8
Hookworm	1/9	1/8

Co-infections are depicted as number of participants infected out of total number of participants tested.

including CD24^{hi}CD38^{hi}, CD24^{hi}CD27$^+$ or CD1d^{hi}(CD5$^+$) (Fig. 6A). No differences were found between infected or uninfected donors for CD24^{hi}CD38^{hi} or CD24^{hi}CD27$^+$ B cells (Fig. S4). However, significantly higher percentage of CD1d^{hi} B cells was found in infected children compared to uninfected children (Fig. 6B). There was also a trend for more CD1d^{hi}CD5$^+$ B cells in infected children, however, the total numbers were so low (< 1 %) that the reliability of this measurement can be questioned. Importantly, after six months of treatment with praziquantel, CD1d^{hi} percentages were reduced to levels comparable to the uninfected control group (Fig. 6B). Of note, CD1d^{hi} levels in the uninfected control group were significantly increased over the same period, which may reflect seasonal changes. Alternatively, 6 out of 8 donors from the uninfected group had an increased population of plasmablasts (CD19$^+$CD24^{lo}CD38^{hi} cells; Fig. S5), suggesting a recent, unrelated infection in Lambaréné, but not in the nearby village (~15

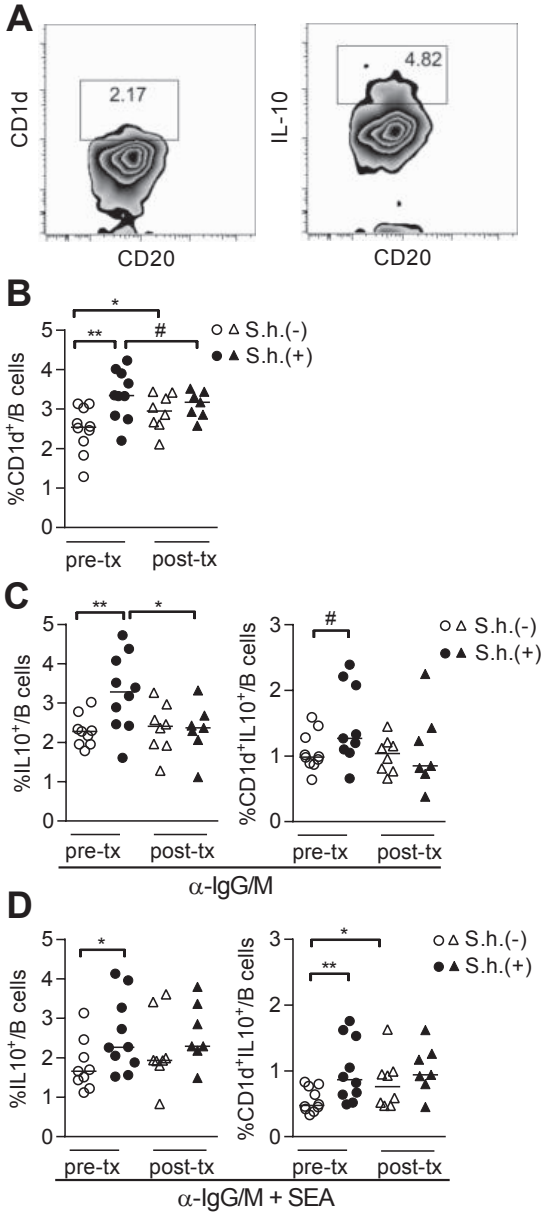


Figure 6. Presence of IL-10-producing CD1d^{hi} B cells during *S. haematobium* (S.h.) infection.
(A) The CD1d^{hi} B cells and the IL-10 production of the total B cells were gated according to the gating strategy depicted in this graph. The gating of the IL-10 production of the different Breg subsets is similar as the total B cell gating. (B) PBMC were fixed and stained for Breg markers, including CD1d, and analyzed by flow cytometry. (C) Total peripheral blood B cells were cultured with anti-IgG/IgM or (D) anti-IgG/IgM plus SEA (10 µg/ml) for two days. Intracellular IL-10 production of the total B cells and the CD1d^{+} B cells was determined following PMA/Ionomycin/LPS and BrefA stimulation.

km, PK15), which may have caused the increase in CD1d^{hi} B cells observed in the uninfected children.

To compare the IL-10 production in B cells from infected and uninfected children, total peripheral blood B cells were stimulated with anti-IgG/IgM. Subsequent intracellular analysis showed an increase in IL-10-producing B cells in the total B cell population of infected children, which was significantly reduced after treatment (Fig. 6A,C). Further gating on the CD1d^{hi} B cell subset, confirmed that in particular CD1d^{hi} B cells from infected children produce more IL-10 as compared to uninfected children (Fig. 6C). Interestingly, we observed a slightly different pattern, when the cells were stimulated with anti-IgG/IgM plus SEA (Fig. 6D). Although also in this condition, both total and CD1d^{hi} B cells from infected children produce significantly more IL-10, this was not downregulated upon treatment. This may reflect the presence of a small, but persistent population of schistosome-specific B cells in the circulation of previously infected children that more readily produce IL-10 in response to SEA, as its cognate antigen, compared to the more aspecific stimulation with antibodies directed against IgG/IgM. Although it cannot be excluded that SEA may also act as an adjuvant, this seems to be less likely as only little IL-10 inducing activity of SEA is found in B cells from uninfected children. The fact that somewhat elevated IL-10 levels in anti-IgG/IgM plus SEA-stimulated B cells from uninfected group at post-treatment are observed compared to pre-treatment may again reflect seasonal effects or recent unrelated infections, suggested by the enhanced population of plasmablasts in this group (Fig. S5).

Altogether, these data confirm that also in humans, schistosomes can induce IL-10-producing Breg cells, of which the CD1d^{hi} B subset is the most prominent. Therefore schistosomes can be recognized as powerful Breg cell inducer in both mice and humans.

Discussion

In this study we provide evidence that during chronic schistosomiasis IL-10-producing CD1d^{hi} B cells are induced in both humans and mice. In mice, schistosome-induced IL-10-producing Breg cells were necessary for protection against AAI as shown here in chimeric IL-10-/- B cell mice. Splenic B cells were the most prominent source for IL-10. Interestingly, the induction of IL-10-producing splenic B cells by infectious agents has been previously documented for *L. major* [12], murine cytomegalovirus [23] and for *Schistosoma mansoni* by our group and by Amu et al [7]. These studies favor the concept that chronic infections, drive strong immunoregulatory processes in which IL-10-producing B cells seem to be important players (reviewed in [24]).

However, several reports have indicated that B cells can also suppress inflammation via IL-10-independent mechanisms. In a colitis model, mesenteric B cells were capable of reducing CD4^{+} T cell-dependent colon inflammation [25] and mesenteric CD23^{hi} B cells of *H. polygyrus*-infected mice were capable of inhibiting inflammation both in an EAE model and in an HDM-specific AAI model via an unknown mechanism [26]. Furthermore, studies in *S. mansoni*-infected µMT mice, where the lack of B cells led to increased liver pathology have suggested the involvement of FcR-dependent mechanisms [13]. Although their mode of action is still unknown, we describe here that transferred pulmonary B cells also protected against AAI independently of IL-10 and

Treg cells. The putative role of antibodies or interaction with FcR has not been studied yet.

Further, we characterized the phenotype and function of the dominant IL-10-producing B cell subset, finding a regulatory function for CD1d^{hi} MZ B cells in the spleen of schistosome-infected mice. These data are in line with several other studies in auto-immunity models pointing towards MZ B cells as regulators of type 1 inflammation in SLE, ACAID or CHS [25,27]. In addition, it was shown that CD1d^{hi}-expressing splenic B cells reduced inflammation in a chronic colitis model [25] and AAI during 'worm only'-schistosome infection [7]. However, within the spleen, other regulatory subtypes have been suggested; a rare CD1d^{hi}CD5^{+} B cell subset, termed B10 cells, which are capable of down-regulating inflammatory responses in a number of different auto-immune or contact hypersensitivity models [15] and transitional type 2 MZ precursor B cells (CD21^{hi}CD23^{hi}IgM^{hi}), described in mouse models for CIA [22,25] and SLE [28]. It is not fully clear whether these B cells are complete unique subsets because there is a substantial overlap between the (co-)expression of various markers, such as CD1d, CD5, CD21, CD23 and IgM. In addition, local inflammation or chronic infection may change the expression of individual markers complicating distinctions between the different proposed cell subsets.

Here we show that helminth-induced MZ B cells not only reduced allergic inflammation via IL-10 but also via the induction/recruitment of active FoxP3^{+} Treg cells. In humans, Treg cells are known to be an important element in reducing allergic inflammation as a mutation in the FOXP3 gene is associated with severe eczema, food allergy and high levels of IgE and eosinophilia [29]. Furthermore, children with asthma show quantitative and functional impairment of CD4^{+}CD25^{+} Treg cells in the BAL fluid[30], while children that have outgrown their allergy have increased frequencies of allergen-responsive Treg cells [31,32]. Therefore, having a system that embraces not only one regulatory system but in fact two seems to be a very efficient strategy to develop tolerance to environmental stimuli and prevent allergy. Indeed, in several studies Breg function has been linked to induction or recruitment of Treg cells, i.e. in a model of ACAID, colitis, EAE, SLE [24,25] and allergic inflammation [7]. Importantly, in these studies including the one presented here, Breg-induced immune regulation does not fully depend on Treg cell activity, with the exception of the study by Amu et al. [7], where in fact an excess of regulatory CD1d^{hi} B cells was transferred to recipient mice on three consecutive days, in contrast to our study with only one B cell injection before the challenge.

Although the majority of studies on Breg cells have been conducted in mouse models there are now a few reports that confirm the existence of human Breg cells, in which both equivalents of already described 'mouse' Breg cells are identified in addition to some new subsets. Correale et al. have reported human IL-10-producing CD1d^{hi} B cells in helminth-infected MS patients [18]. However, these patients were infected with a mixture of different helminth species. Here, we now have established a causal relation between a single species of helminth, schistosomes and increased levels of IL-10-producing CD1d^{hi} B cells, which were reduced to 'normal' levels after anti-schistosome treatment. Of note, in both schistosome-infected and uninfected children, other geohelminths were found and for ethical reasons both groups were treated with the antihelmintic albendazole in addition to treatment with praziquantel for the schistosome-infected group. Despite their initial presence, we find reduced numbers of CD1d^{hi} B cells after treatment only in the schistosome-infected group but not in the

schistosome-uninfected group. These data suggest that schistosomes have a more dominant effect on Breg cell induction than other gut-associated helminths. So far, two other human Breg subsets have been described, namely $CD24^{hi}CD27^{+}$ [17] and $CD24^{hi}CD38^{hi}$ Breg cells in healthy individuals, of which the activity of the latter was impaired in SLE patients [16]. Interestingly, we did not observe differences in these two Breg cell subsets in peripheral blood of infected versus uninfected children, indicating that schistosomes primarily induce $CD1d^{hi}$ Breg cells. This discrepancy suggests that the various Breg cell subsets described so far may require different conditions for their development and activation.

As illustrated above, evidence from animal studies and a few human studies points towards a significant role for IL-10-producing Breg cells in modulating pathogenic hyperinflammatory responses. As such, it would be of great therapeutic interest if Breg cell activity could be specifically induced. For this, helminth infections may be of particular value, as in vitro exposure of splenic B cells to live schistosome worms or peritoneal injection of schistosome egg-derived glycans or filarial glycoproteins induces IL-10-producing B cells [7,33]. However, the identification of the exact helminth-derived molecules involved, is a critical step as enhanced activity of Breg cells may form a valuable new target for therapy of rhinitis and/or allergic asthma.

Materials and methods

Ethics statement

Mice were housed under SPF conditions at the animal facilities of the Leiden University Medical Center in Leiden, the Netherlands. All animal studies were performed in accordance with the guidelines and protocols (DEC-07062, 07152, 08034, 09141) approved by the Ethics Committee for Animal Experimentation of the University of Leiden, The Netherlands. The human study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the "Comité d'Ethique Regional Independent de Lambaréné" (CERIL). Written informed consent was obtained from parents or legal guardians of children participating in the study.

Animals

Six week-old female C57/Bl6 OlaHsd mice were purchased from Harlan. For the generation of chimeric IL-10-producing B cell-deficient mice, B cell-deficient μMT mice were irradiated to remove the bone marrow (BM). Subsequently, the irradiated mice were reconstituted with 80% of μMT BM and 20% $IL\text{-}10^{-/-}$ BM cells (IL-10-deficient) or with 80% of μMT BM and 20% wild-type (WT) BM as described [10]. DEREG (DEpletion of REGulatory T cells) mice were kindly provided by Dr. T. Sparwasser [19].

Parasitic infection and AAI induction

Mice were infected percutaneously with 40 *S. mansoni* cercariae and the infection lasted until 14 weeks (chronic phase starts around week 12) [8]. For AAI induction, mice were sensitized twice by i.p. injections of OVA (10 μg/mL, Worthington Biochemical Corp) in Imject Alum (2

mg/ml; Pierce) at 17 and 10 days before challenge. Sensitization was initiated during week 11 and 12 after the start of infection. Ten days after the last injection, mice received OVA aerosol challenges (10 mg/ml in PBS) for three consecutive days in the 14th week of infection. Mice were sacrificed 24 hours after the last challenge. BAL fluids were collected and phenotyped by flow cytometry[8].

Study population

Venous blood was obtained from 20 school children living in Lambaréné (Gabon) or from a nearby village (PK15) where *Schistosoma haematobium* is endemic. *S. haematobium* infection was detected by examining 10 ml of urine passed through a 12-µm-poresize filter (Millipore) and the eggs were stained with a ninhydrin solution. Children were classified as 'infected' if at least one *S. haematobium* egg was detected in the urine or 'uninfected' if three consecutive urine samples were negative. Infections with intestinal helminths *A. lumbricoides, T. trichiura* and hookworm were determined by analyzing one fresh stool sample using the Kato-Katz method [20]. Infection with *P. falciparum* was determined by PCR [21]. *S. haematobium*-infected children were treated with three doses of praziquantel (40 mg/kg) every two months. Intestinal helminth and malaria infected children received respectively a single dose of albendazole (400 mg) and an artemisinin-based combination therapy as per the local guidelines.

Mouse cell purification and cell sorting

Single cell suspensions were prepared from the spleens, mesenteric lymph nodes, and mediastinal lymph nodes by dispersion through a 70-µm cell sieve (Becton Dickinson). Perfused lungs were minced to ~1 mm pieces and digested by collagenase II/Dnase for 1 hour. The digested lungs were sequentially dispersed through 70- and 40-µm sieves. Erythrocytes were removed from the spleen and lung single cell suspensions by lysis. B cells were purified using anti-CD19 MicroBeads (Miltenyi Biotec). Follicular B cells (CD21^{int}CD23^{hi}) and marginal zone B cells (CD21^{hi}CD23$^{int/low}$) were stained with antibodies against CD19-PE (MB19-1, eBioscience), CD21-APC (7G6, BD Pharmingen), and CD23-FITC (B3B4, eBioscience) and separated using FACSAriaII cell sorting (Becton Dickinson). The sorted subsets were routinely ~95% pure. For the depletion of MZ B cells, splenocytes were first incubated with CD19-PE (6D5, Miltenyi Biotec), followed by anti-PE multisort beads (Miltenyi Biotec) to isolate the B cells. Next, these beads were enzymatically removed and the isolated B cells were incubated with CD21-FITC (7G6, BD Pharmingen) antibody for 20 minutes. The CD21^{hi} MZ B cell fraction was depleted using anti-FITC magnetic beads (Miltenyi Biotec). For the total CD19^{+} B cells, the CD21^{hi} B cells were added back to the CD21^{neg} B cells (mock depletion). The depletion of MZ B cells was ~92% pure. CD4^{+}CD25^{-} T cells were enriched using anti-CD4 and anti-CD25 MicroBeads with a purity of 96% (Miltenyi Biotec).

Human B cell isolation and characterization

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from 20 ml of heparinized blood. B cells were isolated with anti-CD19 MicroBeads (Miltenyi Biotec) with a purity of ~95%. For immunophenotyping different B cell subsets, isolated PBMCs were fixed in

2.4% PFA and stained for CD19-PB (HIB19, eBioscience), CD1d-PE (51.1, eBioscience), CD5-APC (UCHT2, BD), CD24-PeCy7 (ML5, ITK Diagnostics), CD27-APCeFluor780 (O323, eBioscience), and CD38-FITC (HIT2, BD).

Adoptive transfer of isolated mouse B cells

Recipient mice were sensitized with two injections of OVA at day 0 and day 7. Ten days after the last injection, the OVA-sensitized animals received i.v. injection of 5 x 10^6 pulmonary, mesenteric, splenic total or splenic $CD19^+$ B cells depleted for MZ B cells from OVA sensitized-uninfected or OVA sensitized-infected mice. Blocking anti-IL-10R antibody (250 µg; kindly provided by Schering Plough Biopharma) or isotype control antibody was given i.p., one day before adoptive transfer. DEREG mice, which carry a Diphtheria toxin receptor-eGFP transgene under the control of an additional Foxp3 promoter, were treated with two diphtheria toxin (DT, 1 µg/ml) i.p. injections: one day before and two days after the adoptive transfer of B cells in order to deplete the $FoxP3^+$ Treg cells. After two days, mice were challenged for three consecutive days and sacrificed 24 hours after the last challenge.

In vitro mouse B cell stimulation and co-culture with $CD4^+CD25^-$ T cells

Mouse $CD19^+$ B cells and B cell subsets (1 x 10^5 cells) were cultured in the presence of SEA from *S. mansoni* eggs (20 µg/mL) for five days. Supernatants were stored for later cytokine analysis by ELISA. For in vitro Treg induction, B cells (1 x 10^5 cells) were first irradiated with 2600 RAD and subsequently, co-cultured with $CD4^+CD25^-$ T cells (1 x 10^5 cells) in the presence of medium or anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml). An isotype control anti-β-gal (10 µg/ml), or anti-IL-10 receptor (10 µg/ml) was added. After five days, cells were fixed according to the eBioscience FoxP3 fixation/permeabilization kit. Proliferation was confirmed by cell counts. Cytokines were measured in the cell culture supernatant using Luminex or ELISA (IL-1β,-4,-5,-6, -10, -12p40/70, -13, IFN-γ, TNF-α).

Human B cell stimulation and intracellular staining for IL-10

Freshly isolated B cells (1 x 10^5) were stimulated for 48 hours with 2.5 µg/ml anti-human IgG/IgM (Jackson ImmunoResearch) in the presence or absence of 10 µg/ml SEA from *S. haematobium* eggs. For ICS of IL-10, B cells were restimulated with PMA (50 ng/ml), ionomycin (2 µg/ml), and LPS (100 ng/ml; Invivogen) for 6 hours with the final 4 hours in the presence of BrefA (10 µg/ml; Sigma-Aldrich), followed by fixation with FoxP3 fixation/permeabilization kit and stained for CD1d-PE (51.1, eBioscience), CD20-APCeFluor780 (2H7, eBioscience), and IL-10-biotin (JES3-12G8, Abd Serotec) followed by second incubation with streptavidin-Qdot525 (Invitrogen).

Statistical analysis

All murine results are expressed as mean ± SEM and the different groups were tested using the Student's t-test (two-tailed). Differences between infection groups in humans were tested by the Mann-Whitney U test. Differences within the same group pre- and post-treatment were compared by Wilcoxon matched pairs test. Probability values less than 0.05 were considered significant. *** $p< 0.001$, ** $p<0.01$, * $p<0.05$ and # $p<0.1$.

Acknowledgements

We thank the study participants from Lambaréné and PK15 in Gabon for volunteering to participate in this study and all the field workers involved.

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Supplementary material

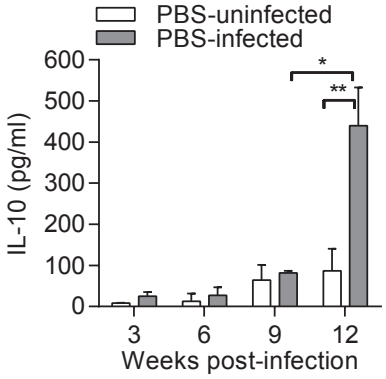

Figure S1. IL-10 production by CD19^{+} B cells during infection.
Mouse CD19^{+} B cells were isolated from the spleen at different time points during *Schistosoma mansoni* infection. The B cells were cultured in the presence of SEA from *S. mansoni* eggs (20 µg/mL) for five days. Supernatants were stored for IL-10 analysis by ELISA. This experiment represents one experiment with 3-4 mice per group.

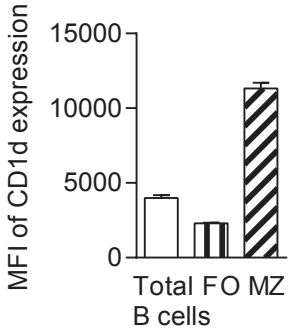

Figure S2.
Geometric mean of CD1d fluorescence intensity on total B cells, FO and MZ B cells.

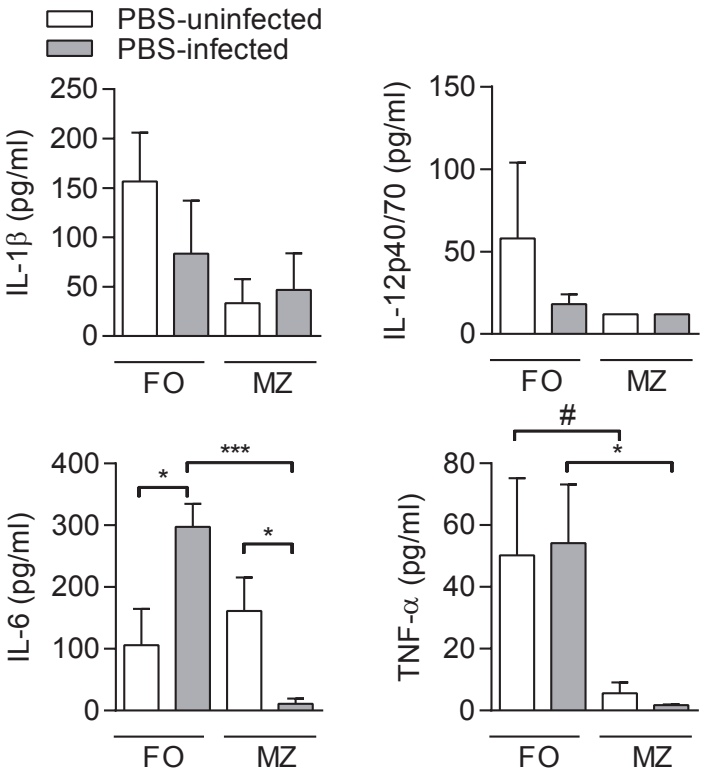

Figure S3. Production of cytokines after SEA stimulation.
MZ and FO B cells from PBS-uninfected and PBS-infected were sorted using flow cytometry and cultured for five days in the presence of SEA for IL-10 production as presented in Fig. 2A. In addition, we measured IL1-β, IL-12p40/70, IL-6 and TNF-α using Luminex.

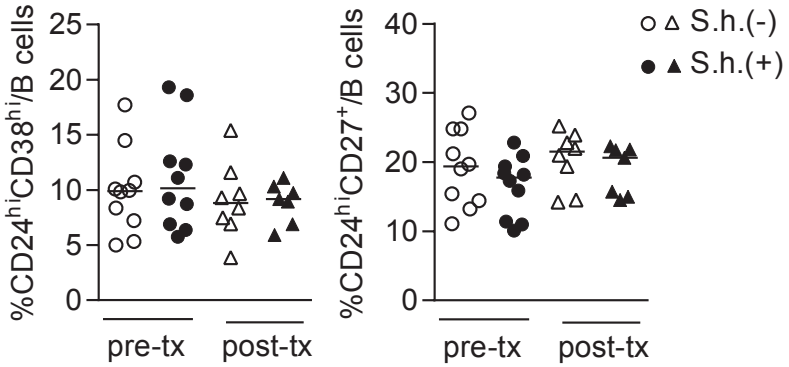

Figure S4.
Percentage of CD24^{hi}CD38^{hi} and CD24^{hi}CD27^{+} B cells in peripheral blood of Gabonese children pre- and post-treatment, performed as described in legend to Fig. 6B.

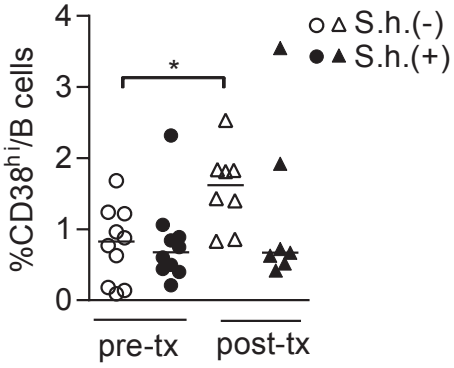

Figure S5.
Percentage plasmablasts in peripheral blood of Gabonese children pre- and post-treatment, performed as described in legend to Fig. 6B.

7

Alterations in Peripheral Blood B Cell Subsets and Dynamics of B Cell Responses During Human Schistosomiasis.

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Author Summary

Schistosomiasis affects over 200 million people and especially children in developing countries. It causes general hyporesponsiveness of the immune system, which until now has predominantly been described for various T cell subsets as well as dendritic cells. B cells in this context have not yet been investigated. To address this question we phenotyped B cell subsets present in peripheral blood from *S. haematobium* infected and uninfected schoolchildren living in an endemic area in Lambaréné, Gabon. Children with schistosomiasis had an increased frequency of various memory B cell subsets, including subsets associated with B cell exhaustion, and a concomitant decrease in naïve B cells. To study the effect of *Schistosoma* infection on B cells in more detail we isolated peripheral blood B cells and found that B cells from infected children had a reduced capacity to proliferate and produce TNF-α in response to both B cell receptor and Toll-like receptor stimulation. These results provide new insights into the role of B cells in the host immune response to schistosomiasis and may provide a novel target for therapeutic strategies.

Abstract

Antibody responses are thought to play an important role in control of *Schistosoma* infections, yet little is known about the phenotype and function of B cells in human schistosomiasis. We set out to characterize B cell subsets and B cell responses to B cell receptor and Toll-like receptor 9 stimulation in Gabonese schoolchildren with *Schistosoma haematobium* infection. Frequencies of memory B cell (MBC) subsets were increased, whereas naïve B cell frequencies were reduced in the schistosome-infected group. At the functional level, isolated B cells from schistosome-infected children showed higher expression of the activation marker CD23 upon stimulation, but lower proliferation and TNF-α production. Importantly, 6-months after 3 rounds of praziquantel treatment, frequencies of naïve B cells were increased, MBC frequencies were decreased and with the exception of TNF-α production, B cell responsiveness was restored to what was seen in uninfected children. These data show that *S. haematobium* infection leads to significant changes in the B cell compartment, both at the phenotypic and functional level.

Introduction

Schistosomiasis is a major parasitic disease of humans in the developing world, with over 200 million people infected worldwide [1]. As with other chronic helminth infections, schistosomes cause widespread immune activation and deregulation leading to general T cell hyporesponsiveness supporting the long term survival of the parasite and minimizing immunopathology [2–4]. Resistance to schistosomiasis is only gradually acquired and is attributed to cumulative exposure to infection [5,6]. Mice vaccination experiments with radiation-attenuated *S. mansoni* cercariae showed less protection against re-infection in μMT B cell-deficient mice than in wild-type mice [7], and the transfer of serum from infected rodents to naïve animals can protect against infection [8,9], suggesting that antibodies are important for protection against infection. In human infection, protective IgA, IgE and IgG levels have been demonstrated against adult worm antigens [10,11], and resistance to (re-) infection is correlated with an increased ratio between IgE and IgG4 [12]. Furthermore, expression of CD23, the low affinity IgE receptor which can be strongly up-regulated by IL-4 [13], is also correlated with development of resistance to *Schistosoma* re-infection [14,15]. While B lymphocytes support the establishment of the strong Th2 profile associated with helminth infections [16], more recently they have also been shown to play an active regulatory role in the course of *Schistosoma* infections [17] mostly effecting T cell responses.

In general, immunological memory is characterized by its ability to respond more rapidly and robustly to re-infection and is dependent on the generation and maintenance of memory B cells (MBCs) [18]. Memory B cells, originally defined as $CD27^+$ [19], can be further characterized into additional subsets by co-staining with IgD into non-switched MBCs ($CD27^+IgD^+$), switched MBCs ($CD27^+IgD^-$) and double negative MBCs ($CD27^-IgD^-$) [20]. Furthermore, co-staining with CD21 can be used to separate classical MBCs ($CD27^+CD21^+$) from activated MBCs ($CD27^+CD21^-$) and atypical MBCs ($CD27^-CD21^-$) [21]. Based on these markers, naïve B cells can be classified as $CD27^-IgD^+$ or $CD27^-CD21^+$. Recent studies have shown that chronic HIV infection [21,22] as well as exposure to and infection with *P. falciparum* malaria [23,24] are associated with the expansion of atypical or 'exhausted' MBCs ($CD27^-CD21^-$). These cells are characterized by high expression of the inhibitory receptor FCRL4 [25,26], and it has been suggested that this population may contribute to diminished pathogen-specific antibody responses in infected individuals. Other chronic infections such as hepatitis C virus (HCV) [27] have also shown perturbations in the distribution of peripheral B cells subsets, most notably within the memory B cell compartment suggesting that MBCs may play a role in disease pathogenesis as well as insufficient immune response to combat the disease.

Ligation of the B cell receptor (BCR) by its cognate antigen leads to the production of antibodies and, depending on the cytokines produced by Th cells, to further antibody isotype switching and affinity maturation. B cells can also express a variety of innate receptors, most notably Toll-like receptors (TLRs), and can play a significant role in innate immune responses as B cells upregulate activation markers, proliferate and secrete cytokines upon engagement of these receptors [28,29]. Importantly, TLR stimulation can also potentiate the T cell-dependent production of antibodies [30,31]. TLR9 is highly expressed in human B cells and is ligated by bacterial DNA motifs containing unmethylated CpG dinucleotides. Previous studies have clearly

demonstrated that TLR9 stimulation is sufficient to directly induce both naïve and memory B cell proliferation and activation [32,33]. In addition, the role of B cells in innate immune responses has gained further interest as several studies have demonstrated a pathogenic role for B cells independent of their ability to produce antibodies. For example, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are associated with abnormally increased pro-inflammatory cytokine production by B cells, including TNF-α. Importantly TNF-α [34], along with more recently IL-17 [35], is also one of the key cytokines involved in schistosome-induced pathology [36,37].

Currently there is little information on the composition of the peripheral blood B cell compartment or the concomitant adaptive and innate functionality of B cells during the course of human *Schistosoma haematobium* infection. In this study, we compared the circulating B cell subsets in peripheral blood of schistosome-infected and uninfected Gabonese schoolchildren and their B cell response to BCR and TLR engagement.

Results

Study population characteristics

We recruited *S. haematobium*-infected (N = 29) and -uninfected (N = 27) schoolchildren (8-16 years old) for phenotypic B cell analysis. From these, we selected 10 from each group for more extensive immunological analyses and performed follow-up studies six months later on 7 infected children treated with 3 rounds of praziquantel and 8 uninfected children. Following treatment all *S. haematobium*-infected children were infection free. As described in Table 1, there were no significant differences between the two groups in the prevalence and infection intensity of other parasitic infections such as *P. falciparum*, *A. lumbricoides*, *T. trichiura* or hookworm. Furthermore, age and gender were comparable between the two groups.

Table 1: Characteristics of the study population.

	***S. haematobium* infected**	***S. haematobium* uninfected**	**p value**
N	29	27	
Age in years (mean (SD))	11.36 (2.46)	11.7 (1.82)	0.887*
Male/female	18/11	14/13	0.590#
Egg counts (median (range))	11 (1-1000)	0	0.000^
Co-infections			
Plasmodium falciparum	5/28	1/26	0.194#
Ascaris lumbricoides	5/23	5/23	1.000#
Trichuris trichiura	6/23	10/23	0.353#
Hookworm	3/23	1/20	0.610#

Co-infections are depicted as number of participants infected out of total number of participants tested. * independent student's T test; # Fisher's exact test; ^ Mann-Whitney U test.

Serum immunoglobulin levels

In schistosomiasis, resistance is acquired slowly and it is not clear how the B cell compartment and B cell function are affected. To gain insight into global B cell function during *S. haematobium* infection we studied immunoglobulin isotypes and IgG subclasses in serum. Consistent with previously published data [43], IgG4 levels were increased in *S. haematobium*-infected compared to uninfected children and were significantly reduced following praziquantel treatment (Figure 1). No significant differences were observed in serum IgM, IgG1, IgG2, IgG3, IgA and IgE between the groups.

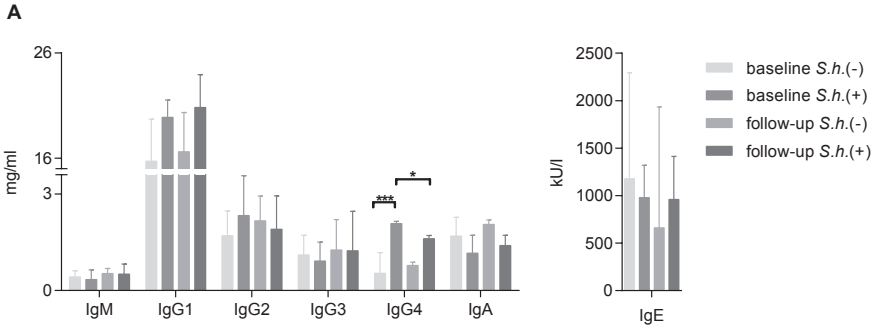

Figure 1. Serum immunoglobulin analysis.
Serum samples were analyzed for total human IgM, IgG1, IgG2, IgG3, IgG4, IgA by Luminex and IgE by ELISA. Bars represent median with interquartile range. Number of donors in each group: baseline *S.h.* –ve n = 9, baseline *S.h.* +ve n = 10, follow-up *S.h.* –ve n = 8 and follow-up *S.h.* +ve n = 7.

B cell inflammatory cytokine response, activation and proliferation

To address whether B cell function was altered during *Schistosoma* infection, we measured in vitro cytokine responses, proliferation and activation markers of peripheral blood B cells in response to BCR (anti-IgG/IgM) and TLR9 (CpG) signaling by flow cytometry in uninfected and infected children. We first focused on responses in uninfected children. Intracellular production of the pro-inflammatory cytokine TNF-α and expression of surface CD23, an indicator of TLR activation [44], were significantly induced by BCR and TLR9 engagement; dual receptor engagement did not further increase these levels (Figure 2A, B and S1A, B). Intracellular Ki-67, a marker of proliferation, was not induced by BCR stimulation alone, but was increased following CpG stimulation, and as expected [45], dual BCR and TLR engagement was required for optimal B cell proliferation (Figure 2C and S1C). Frequencies of unstimulated B cells that produced TNF-α, expressed CD23 or were positive for Ki-67 did not differ between infected and uninfected children. As TNF-α production and CD23 expression levels were highest following CpG stimulation, with no significant enhancement when combined with anti-IgG/IgM co-stimulation, we focused on CpG stimulations for comparison between infected and uninfected children. TNF-α-producing B cell frequencies were significantly lower in infected children as compared to uninfected children (Figure 2D), and this was not upregulated upon treatment. In

contrast, CD23-expressing B cell frequencies were significantly elevated (Figure 2E) and Ki-67$^+$ B cells significantly reduced (Figure 2F) in the infected children; both were restored following praziquantel treatment to levels observed in the uninfected children. Baseline frequencies of CD23$^+$ B cells in *ex-vivo* PBMCs did not differ between schistosome-infected children and healthy controls, 33.9% versus 40.6% respectively (p = 0.932; data not shown). Taken together, these data suggest that *Schistosoma* infection leads to alterations in B cell responses, and that some of these changes are long lasting and persist at least six months after removal of infection.

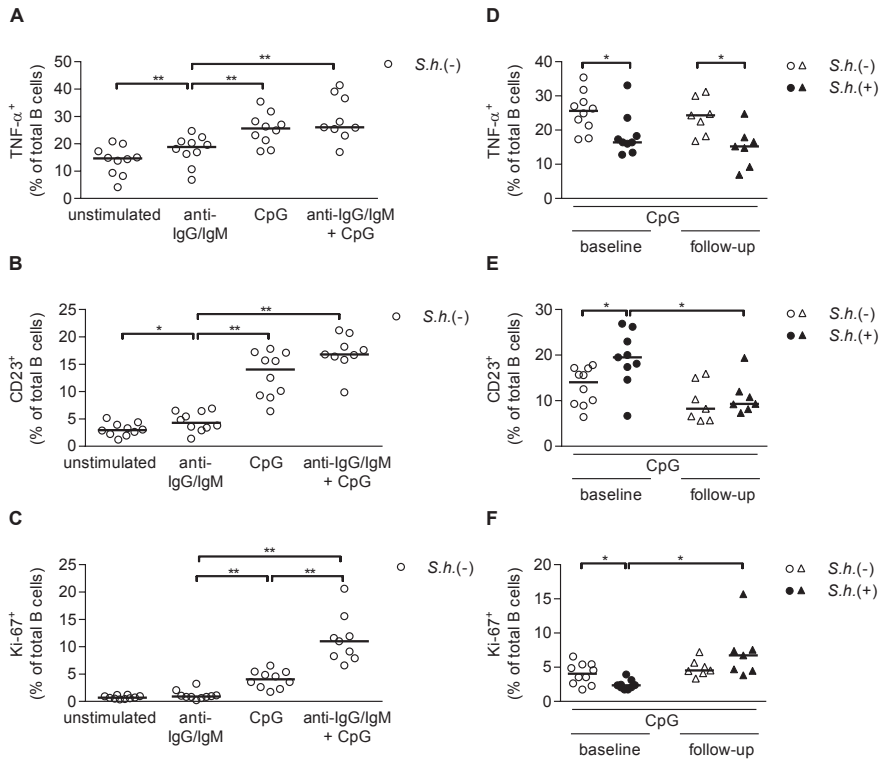

Figure 2. B cell inflammatory cytokine response, activation and proliferation.
Total peripheral blood B cells were cultured with anti-IgG/IgM (2.5 µg/ml), CpG (5 µg/ml) or anti-IgG/IgM plus CpG for two days, restimulated with PMA/Ionomycin/LPS and BrefA and fixed. Levels of intracellular TNF-α (A), CD23 expression (B) and intracellular Ki-67 (C) were measured in *S. haematobium*-uninfected children by flow cytometry. Levels of intracellular TNF-α (D), CD23 expression (E) and intracellular Ki-67 (F) following CpG stimulation in infected and uninfected children at baseline and follow-up. Horizontal bars represent median. Number of donors in each group: baseline *S.h.* –ve n = 10, baseline *S.h.* +ve n = 9, follow-up *S.h.* –ve n = 7 and follow-up *S.h.* +ve n = 7.

B cell subpopulation analysis

To further explore schistosome-induced alterations in the B cell compartment, we next compared circulating B cell subsets in peripheral blood between infected and uninfected children by flow cytometry. No statistically significant differences in the proportion of total peripheral B cells

were found between schistosome-infected children and healthy controls, 15.2% versus 13.7% respectively ($p = 0.776$). Four distinct $CD19^+$ B cell subsets can be distinguished by additional expression of CD27 and IgD [20] (Figure 3A). These are defined as naïve B cells ($CD27^-IgD^+$), non-switched MBCs ($CD27^+IgD^+$) also referred to as marginal zone-like B cells, switched MBCs ($CD27^+IgD^-$), and double negative MBCs ($CD27^-IgD^-$). The proportion of switched MBCs (Figure 3B) and double negative MBCs (Figure 3D) was significantly increased in schistosome-infected children and these levels were significantly reduced to levels comparable to the uninfected control group following treatment. Concomitantly there was a trend toward a lower percentage of naïve B cells ($p = 0.062$) (Figure 3E) in schistosome-infected children and following treatment the level of naïve B cells was significantly increased. No differences were observed in the levels of non-switched MBCs (Figure 3C). Interestingly, we noted a positive correlation between total serum IgG4 levels and the percentage of switched MBCs (Spearman $r = 0.407$, $p < 0.05$) as well as a trend with DN MBCs (Spearman $r = 0.330$, $p = 0.056$) and a negative correlation with naïve B cells (Spearman $r = -0.392$, $p < 0.05$).

To further investigate immunoglobulin expression on B cells we evaluated an additional 8 schistosome-infected children and 8 endemic controls with an antibody panel that included a brighter CD27 antibody (Figure S2A). We found similar differences with respect to an increase in the DN MBCs (Figure S2D) and a decrease in naïve B cells (Figure S2E) in schistosome-infected children. Although the switched MBCs (Figure S2B) did not differ significantly in this cohort, when grouped with the original data (from Figure 3) the overall comparison remained significant. We first evaluated immunoglobulin levels on $CD27^+$ B cells [46] and found no differences in the proportion of non-switched IgM^+ MBCs (Figure 4A), or switched IgA^+ (Figure 4B) and IgG^+ (Figure 4C) MBCs between schistosome-infected and -uninfected children. We next assessed immunoglobulin expression on the double negative MBCs ($CD27^-IgD^-$) and while there were no differences in the proportion of either IgM^+ (Figure 4D) or IgA^+ (Figure 4E) DN MBCs, the proportion of IgG^+ DN MBCs was significantly increased in schistosome-infected children (Figure 4F). The majority of the DN MBCs were class-switched (median, 53.7%) with only 8.7% IgM^+ cells, which may potentially be a mixture of naïve and non-switched MBCs, confirming their status as memory B cells. Similar differences in immunoglobulin expression were observed on atypical MBCs (data not shown).

In addition to the classical characterization of the memory B cell subsets, co-staining of CD27 and CD21 [21] can be performed to identify four B cell subsets (Figure 5A): 1) naïve B cells ($CD27^-CD21^+$); 2) activated MBCs ($CD27^+CD21^-$); 3) classical MBCs ($CD27^+CD21^+$) and 4) atypical MBCs ($CD27^-CD21^-$). The proportion of activated MBCs (Figure 5B) was significantly increased and there was a trend toward a higher percentage of atypical MBCs ($p = 0.058$) (Figure 5D) in peripheral blood of schistosome-infected children and levels of both were significantly reduced following clearance of infection. Similarly the level of naïve B cells was significantly increased (Figure 5E) following treatment, while no differences were found in the proportion of classical MBCs either between the groups or at different time points (Figure 5C). While CD10, a marker of immature and germinal center B cells, was not included in these panels separate analysis showed the level of immature and germinal center B cells within our population to be ~2.75% (median) with no differences between schistosome-infected and -uninfected

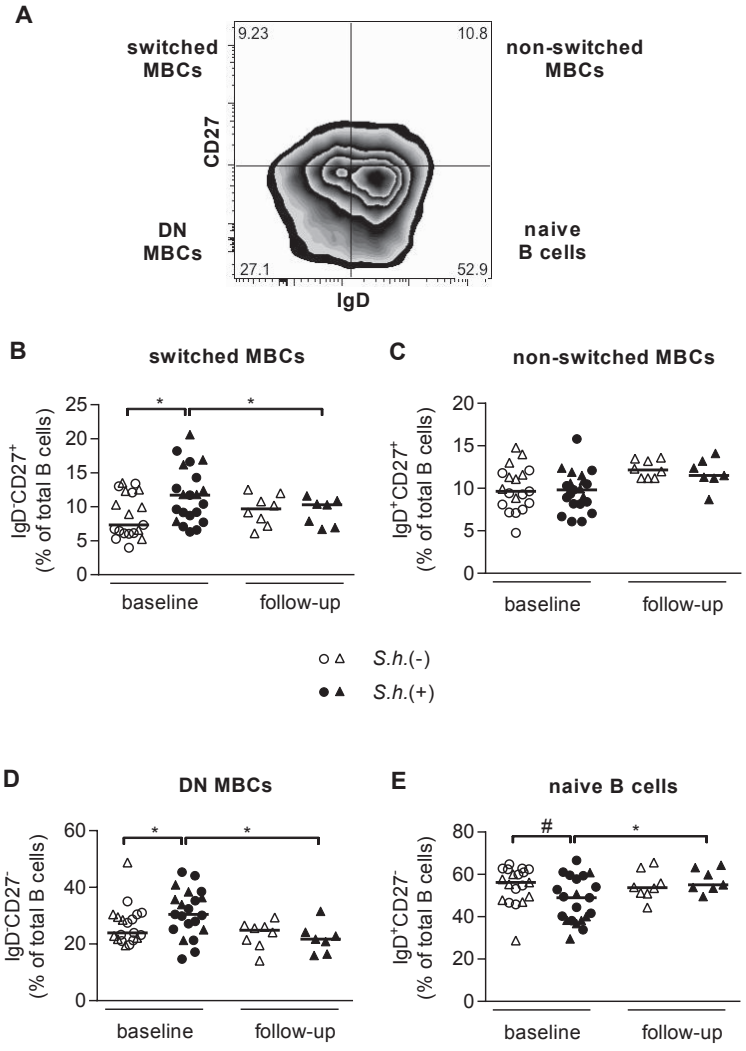

Figure 3. MBC analysis.
PBMC were fixed and stained with B cell phenotyping markers (CD19, CD27 and IgD) and analyzed for B cell subsets by flow cytometry. B cell subset analysis was performed as shown in (A) (representative *S. haematobium*-uninfected child). Proportion of CD19-gated cells that were $CD27^{+}IgD^{-}$ (B, switched MBC), $CD27^{+}IgD^{+}$ (C, non-switched MBC), $CD27^{-}IgD^{-}$ (D, double negative MBC), and $CD27^{-}IgD^{+}$ (E, naïve B cells) were determined for *S. haematobium*-infected and uninfected children at baseline and follow-up. (B, C, D, E) Horizontal bars represent median. Number of donors in each group: baseline *S.h.* –ve n = 19, baseline *S.h.* +ve n = 21, follow-up *S.h.* –ve n = 8 and follow-up *S.h.* +ve n = 7.

children, 2.7% and 2.8% respectively (p = 0.798). To study the nature of atypical MBCs ($CD27^-CD21^-$), we determined the expression levels of HLA-DR and FCRL4, a cell surface marker that is characteristic for exhausted MBCs. In line with previous reports [23,25], FCRL4 was expressed at significantly higher levels on atypical MBCs compared to classical MBCs ($CD27^-CD21^-$) and naïve B cells ($CD27^-CD21^+$) in uninfected children (Figure S3A). Furthermore, HLA-DR expression was significantly higher on atypical MBCs and naïve B cells as compared to classical MBCs (Figure S3B). Both markers were not differentially expressed between B cell subsets of uninfected and infected children (data not shown). Taken together, these data show that *Schistosoma* infection leads to changes in the distribution of peripheral B cell subsets and that praziquantel treatment leads to a reduction of various memory B cell subsets and an increase of naïve B cells.

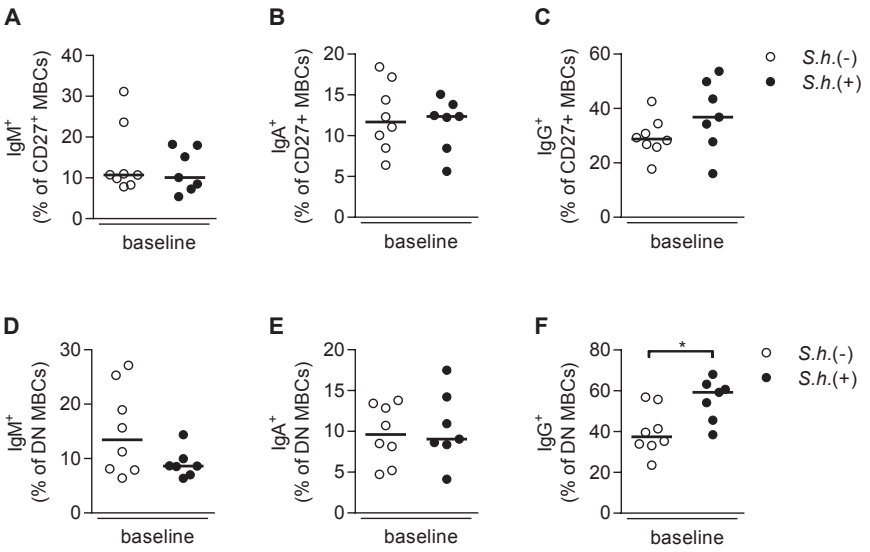

Figure 4. Expression of IgM, IgA and IgG on $CD27^+$ and DN MBCs.
PBMC were fixed and expression of IgM, IgA and IgG on CD27+ B cells was evaluated [46]. Proportions of $CD19^+$ gated cells that were $IgM^+IgD^-CD27^+$ (A), $IgA^+IgM^-IgD^-CD27^+$ (B), and $IgG^+IgM^-IgD^-CD27^+$ (C) were determined for *S. haematobium*-infected and uninfected children at baseline. For immunoglobulin expression on DN MBCs, PBMC were fixed and stained with B cell subset markers (CD19, IgD and CD27) and DN MBCs measured for IgM, IgA and IgG expression. Proportion of $CD19^+CD27^-IgD^-$-gated cells that were IgM^+ (D), IgA^+ (E) and IgG^+ (F) were determined for *S. haematobium*-infected and uninfected children at baseline. Horizontal bars represent median. Number of donors in each group: *S.h.* –ve n = 8 and *S.h.* +ve n = 7.

B cell subset inflammatory cytokine response, activation and proliferation

To investigate whether changes in TNF-α, Ki-67 and CD23 observed in total B cells were attributed to specific B cell subsets, we extended our flow cytometry analysis to include the various B cell subsets defined by CD21 and CD27 expression, first gating on $CD10^-$ B cells. Isolated B cells were stimulated with anti-IgG/IgM, CpG or a combination of the two and stained for the expression of intracellular TNF-α and Ki-67 and surface CD23. We found that CpG stimulation alone or in combination with anti-IgG/IgM resulted in significant loss of CD27 expression (data

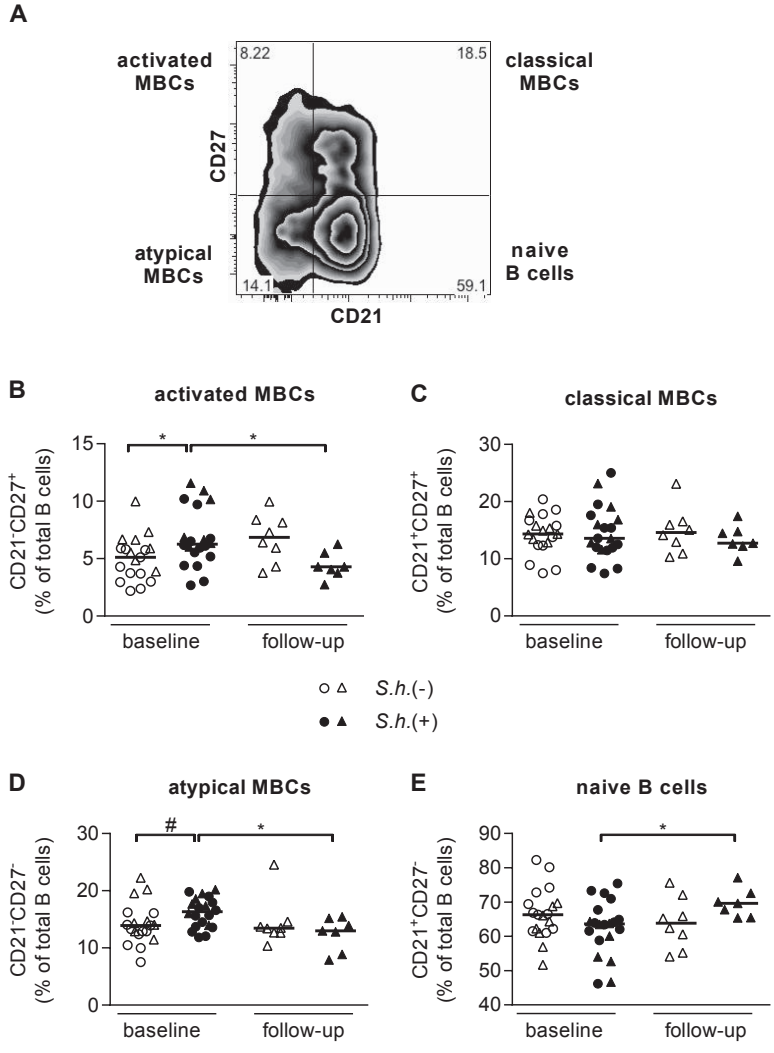

Figure 5. Atypical MBC analysis.
PBMC were fixed and stained with B cell phenotyping markers (CD19, CD21 and CD27) and analyzed for B cell subsets by flow cytometry. B cell subset analysis was performed as shown in (A) (representative *S. haematobium*-uninfected child). Proportion of CD19-gated cells that were $CD27^+CD21^-$ (B, activated MBC), $CD27^+CD21^+$ (C, classical MBC), $CD27^-CD21^-$ (D, atypical MBC), and $CD27^-CD21^+$ (E, naïve B cells) were determined for *S. haematobium*-infected and uninfected children at baseline and follow-up. (B, C, D, E) Horizontal bars represent median. Number of donors in each group: baseline *S.h.* –ve n = 19, baseline *S.h.* +ve n = 20, follow-up *S.h.* –ve n = 8 and follow-up *S.h.* +ve n = 7.

not shown); as a result it was no longer possible to differentiate the four B cell subpopulations with the same criteria as in Figure 5A. We therefore focused on anti-IgG/IgM-stimulated cells, as here we could still distinguish the four B cell populations and analyzed the responses of the various B cell subsets in uninfected children. We found very high frequencies of TNF-α-producing activated MBCs, followed by classical MBCs and atypical MBCs and observed the lowest frequencies among naïve B cells (Figure 6A). When comparing the various B cell subsets for their ability to respond to BCR engagement between infected and uninfected children, we observed significantly less TNF-α$^+$ activated MBCs (Figure 6B) and classical MBCs (Figure 6C) and a trend for lower levels in the naïve B cells and atypical MBCs in the infected children (data not shown). These data reflect the reduced TNF-α production in the total B cell population observed earlier (Figure 2A), and likewise the levels of TNF-α following treatment were not restored to levels observed in the uninfected children for any of the subsets (Figure 6B, C). These data point at a reduced capacity of B cells from infected children to produce TNF-α in response to anti-IgG/IgM stimulation which extends to all B cell subsets studied. While classical MBCs had higher frequencies of CD23 and Ki-67 expressing cells, no differences were observed between infected and uninfected children for any of the subsets or upon treatment.

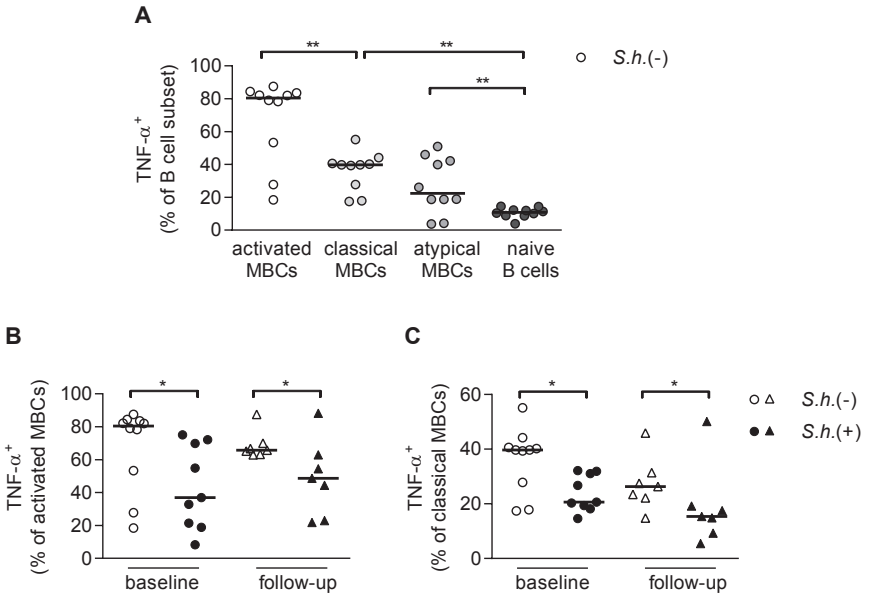

Figure 6. B cell subset inflammatory cytokine response, activation and proliferation.
Total peripheral blood B cells were cultured with anti-IgG/IgM (2.5 µg/ml) for two days, restimulated with PMA/Ionomycin/LPS and BrefA, fixed and stained with B cell subset markers (CD10, CD19, CD21 and CD27) and levels of intracellular TNF-α was measured in *S. haematobium*-uninfected children by flow cytometry (A). Levels of intracellular TNF-α in activated MBCs (B) and classical MBCs (C) in infected and uninfected children at baseline and follow-up. Horizontal bars represent median. Number of donors in each group: baseline *S.h.* –ve n = 10, baseline *S.h.* +ve n = 9, follow-up *S.h.* –ve n = 7 and follow-up *S.h.* +ve n = 7.

Discussion

Although many studies have investigated the types and frequencies of various immune cell subsets, including T cells and DCs [47–49], little is known about the human B cell compartment during the course of *S. haematobium* infection. In the present study, we have analyzed innate and antibody-driving pathways in total B cells and different peripheral B cell subsets and compared their responses between infected and uninfected Gabonese schoolchildren. We found that *S. haematobium* infection leads to changes in B cell function as well as alterations of the total B cell compartment and these changes are not restricted to a single B cell population.

When evaluating B cell functionality, we found significantly lower frequencies of TNF-α-producing and Ki-67$^+$ proliferating B cells in *S. haematobium*-infected children (Figure 2D and F). Interestingly, Ki-67$^+$ B cell frequencies were restored to levels comparable to uninfected children following anti-schistosome treatment (Figure 2F), but TNF-α levels remained significantly lower (Figure 2D). The downregulation of TNF-α and B cell proliferation in *S. haematobium* positive children may be a result of immunomodulation induced by the parasite to prolong its survival. This would be in line with studies showing that TNF-α might play an important role in immunity to helminths: TNF-α production by B cells was necessary for sustained antibody production and establishment of protective immunity to *Heligmosomoides polygyrus* [50]. Moreover, B-cell derived TNF-α has been shown to enhance IFN-γ production by T cells in *Toxoplasma gondii*-infected mice [51] and it is thought that an effective Th1 response is key to natural acquisition of resistance against *Schistosoma* infection [12,52]. Therefore, it is tempting to suggest that the long lasting reduction in B cell capacity to produce TNF-α, as demonstrated post-treatment, may in part contribute to the slow development of resistance to *Schistosoma* infection, although presence of other unknown concomitant viral or fungal infections may also play a role.

In the current study we analyzed CD23 as a TLR activation marker on B cells, however CD23 is also correlated with the development of resistance to *Schistosoma* infection [14]. Furthermore, cross-linking of CD23-bound IgE by antigen may induce cellular activation and increased IgE production [53,54]. Repeated rounds of treatment and *S. mansoni* re-infection led to a gradual increase in CD23 expression and resistance in a cohort of Kenyan children [15]. This seems partly in contrast to our results, as the elevated CD23 levels in infected children are reduced after treatment. However in the Kenyan cohort levels of CD23 expression were evaluated in fresh whole blood samples while here we measured CD23 expression on stimulated B cells as a marker of B cell activation. Baseline levels of CD23 expression on *ex-vivo* PBMCs in our population did not differ, however CD23 levels following treatment were not measured. It would therefore be of interest to measure the dynamics of CD23 expression in our population longitudinally following multiple rounds of treatment.

Perturbations in the frequency of various B cell subsets have been demonstrated in a number of disease states [34] and here we found an increase in the switched MBCs, the double negative MBCs and activated MBCs, as well as a trend toward a higher percentage of atypical MBCs and a concomitant decrease of naïve B cells in schistosome-infected children. All populations were restored to levels observed in uninfected children following treatment. It is unclear whether the increase in naïve B cells following treatment is due to *de novo* generation of B cells from the bone marrow, expansion of the pre-existing naïve B cell population or results

from a decrease in the levels of the other subsets.

It has been shown that in HIV- [21] and malaria-infected individuals [23,24] an exhausted/atypical memory B cell population ($CD27^-CD21^-$), was greatly expanded and that these cells were hyporesponsive and had a decreased ability to differentiate into antibody secreting cells, contributing to the diminished pathogen-specific antibody responses in infected individuals. Likewise, it has been suggested that double negative ($CD27^-IgD^-$) MBCs, which are increased in SLE, might be exhausted/terminally differentiated memory B cells [55,56]. In chronic *S. haematobium* infection we similarly see an expansion of both double negative and atypical MBCs. The overlap between these two MBC subpopulations, their capacity to produce schistosome-specific antibodies or the exact characteristics of their 'exhausted' state are currently not clear.

Nevertheless it is interesting to note that schistosome-infected children carry higher frequencies of IgG^+ double negative ($CD27^-IgD^-$) MBCs compared to uninfected children, whereas no differences are observed in the levels of IgM^+ or IgA^+ DN MBCs (Figure 4). Similarly, no differences were observed in total serum IgM, IgA nor IgG1, IgG2 and IgG3, whereas significant differences were only observed in IgG4 (Figure 1). Interestingly, serum levels of IgG4 were significantly decreased following treatment and correlated with a concomitant decrease in the frequency of the DN MBCs, suggesting that the increase in IgG^+ DN MBCs may be predominantly due to an increase in IgG4-expressing B cells. As IgG4 is associated with susceptibility and IgE with resistance to *Schistosoma* infection, it would be of interest to study these isotypes on the different memory B cell populations in exposed but resistant individuals. These studies could shed further light on the various ways in which *S. haematobium* infection modulates the immune response providing further information for the design of an effective vaccine.

Although the function of double negative and atypical MBCs is not yet clear in the context of *Schistosoma* infection, it is tempting to speculate that these may be expanded as a result of the chronic nature and strong immunomodulation of *S. haematobium* infection. These memory B cells may limit the associated pathology while at the same time limiting the effectiveness of the immune response against the parasite. Indeed, a protective role against malaria infection has been proposed for atypical MBCs through regulation of the host's immune response [23,24]. This parallels $FCRL4^+$ tissue-like MBCs in lymphoid tissue, which may protect against invading pathogens directly or indirectly through the secretion of cytokines and their influence on other immune cell types [25,26]. A recent study has highlighted the dual nature of B cells in immune responses demonstrating that the same B cells may play both a regulatory (IL-10) or pathogenic (IL-6) role depending on the signals received [57]. It would be of interest to investigate concomitantly the production of both IL-6 and IL-10 by the various MBC subsets and naïve B cells to see the balance between pro- and anti-inflammatory B cell responses during *Schistosoma* infection.

As demonstrated above *S. haematobium* infection leads to significant changes in B cell function as well as alterations of the B cell compartment in peripheral blood of infected children as compared to healthy controls. Further studies are needed to define whether these changes in the frequency of the various subsets have functional consequences and what their role is in

the immune response against *S. haematobium*.

Materials and methods

Study population

In April 2008 we initiated a study to investigate the effect of *S. haematobium* infection on B cell phenotype and function. Venous heparinized blood was obtained from 56 school-aged children living in Lambaréné (Gabon), a semi-urban municipality or from its surrounding villages in which *Schistosoma haematobium* infection is endemic and has been previously described in detail [17,38,39]. *S. haematobium* infection was determined prior to blood collection by examining a filtrate of 10 ml of urine passed through a 12-µm-poresize filter (Millipore). Children were classified *S. haematobium*-infected if at least one *S. haematobium* egg was detected in the urine, or uninfected if three consecutive urine samples were negative. Infections with intestinal helminths *Ascaris lumbricoides* and *Trichuris trichiura* were determined by analyzing one fresh stool sample using the Kato-Katz method [40]. Hookworm larvae were determined in a 7-day coproculture of the same stool sample [41]. Infection with *Plasmodium falciparum* was determined by Giemsa-stained thick blood smears [42]. After collection of blood samples, all *S. haematobium*-infected children were treated with a single dose of praziquantel (40 mg/kg) three times every two months. Intestinal helminth- and malaria-infected children received respectively a single dose of albendazole (400 mg) and an artemisinin-based combination therapy as per the local guidelines. The study was approved by the "Comité d'Ethique Regional Independent de Lambaréné" (CERIL; N°06/08). Written informed consent was obtained from parents or legal guardians of all schoolchildren participating in the study.

Cell isolation

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from 20 ml of heparinized blood. B cells were isolated with anti-CD19 MicroBeads (Miltenyi Biotec). The isolated B cells were routinely ~95% pure.

Immunoglobulin assays

Plasma samples were analyzed using the Bio-Plex Pro Assays Immunoglobulin Isotyping Kit (Bio-Rad) for total IgM, IgG1, IgG2, IgG3, IgG4 and IgA levels according to manufacturers' recommendations. Levels of total IgE were measured by ELISA according to manufacturers' instructions (Allergopharma).

B cell stimulation, staining of CD23 and intracellular TNF-α and Ki-67

Freshly isolated B cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Greiner Bio-One), 100 U/ml penicillin (Astellas), 10 µg/ml streptomycin, 1 mM pyruvate and 2 mM L-glutamine (all from Sigma). B cells were seeded at 1 x 10^5 cells per well and stimulated for 48 hours with 2.5 µg/ml anti-human IgG + IgM (Jackson ImmunoResearch), 5 µg/ml CpG ODN 2006 (Invivogen) or anti-IgG/IgM + CpG. To detect intracellular TNF-α, B cells were restimulated with 50 ng/ml PMA (Sigma-Aldrich), 2 µg/ml ionomycin (Sigma-Aldrich), and

100 ng/ml ultrapure LPS (Invivogen) for 6 hours with the final 4 hours in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich), followed by fixation with the FoxP3 fixation/permeabilization kit (eBioscience) and frozen in RPMI supplemented with 20% FCS and 10% DMSO (Merck) and stored at -80ºC until FACS analysis. After thawing, cells were permeabilized (Permeabilization buffer, eBioscience) and labeled with surface anti-CD10-PerCP/eF710 (SN5c, eBioscience), anti-CD20-APC/eF780 (2H7, eBioscience), anti-CD21-FITC (LT21, BioLegend), anti-CD23-PE/Cy7 (EBVCS2, eBioscience), anti-CD27-APC (L128, BD Biosciences), and intracellular anti-Ki-67-eF450 (20Raj1, eBioscience) and anti-TNF-α-biotin (MAB11, eBioscience) followed by second incubation with streptavidin-Qdot525 (Invitrogen).

Characterization of B cells in peripheral blood

For immunophenotyping freshly isolated PBMCs were fixed in 2.4% formaldehyde (Sigma-Aldrich) for 15 minutes at room temperature and, subsequently, frozen in RPMI 1640 medium supplemented with 20% FCS and 10% DMSO and stored at -80ºC until FACS analysis. After thawing, cells were washed and stained for 30 minutes with anti-CD19-PB (HIB19, eBioscience), anti-CD21-FITC (LT21, BioLegend), anti-CD27-APC (L128, BD Biosciences), anti-CD27-APC/eF780 (O323, eBioscience), anti-HLA-DR-APC/Cy7 (L243, BioLegend) and anti-IgD-biotin (IA6-2, BD Biosciences) followed by second incubation with streptavidin-Qdot525 (Invitrogen). Anti-FCRL4-biotin was kindly provided by M. Cooper (Emory University School of Medicine, Atlanta, GA, USA). Alternatively, cells were stained with anti-CD19-BV510 (HIB19, BioLegend), anti-IgA-FITC, anti-IgA-PE (both IS11-8E10, Miltenyi Biotec), anti-CD21-PE/Cy7 (LT21), anti-CD23-APC (EDVCS5), anti-CD27-PerCP/Cy5.5 (L128), anti-CD38-APC/Cy7 (HB7), anti-IgD-PE/CF594 (IA6-2), anti-IgG-PE (G18-145) and anti-IgM-V450 (G20-127) (all from BD Biosciences). Cells were acquired on FACSCanto II and LSR Fortessa flow cytometers (both from BD Biosciences).

Statistical analysis

Differences between study groups were tested using Fisher's exact test for gender and co-infections and by Mann-Whitney U test for *Schistosoma* infection intensity. Age was normally distributed and differences between infection groups were tested using the independent student's T test. Serological and cellular differences between infection groups were tested by the Mann-Whitney U test. Differences within the same group between baseline and follow-up were compared by Wilcoxon matched pairs test. P-values less than 0.05 were considered significant and less than 0.1 a trend. *** $p< 0.001$, ** $p< 0.01$, * $p< 0.05$ and # $p< 0.1$.

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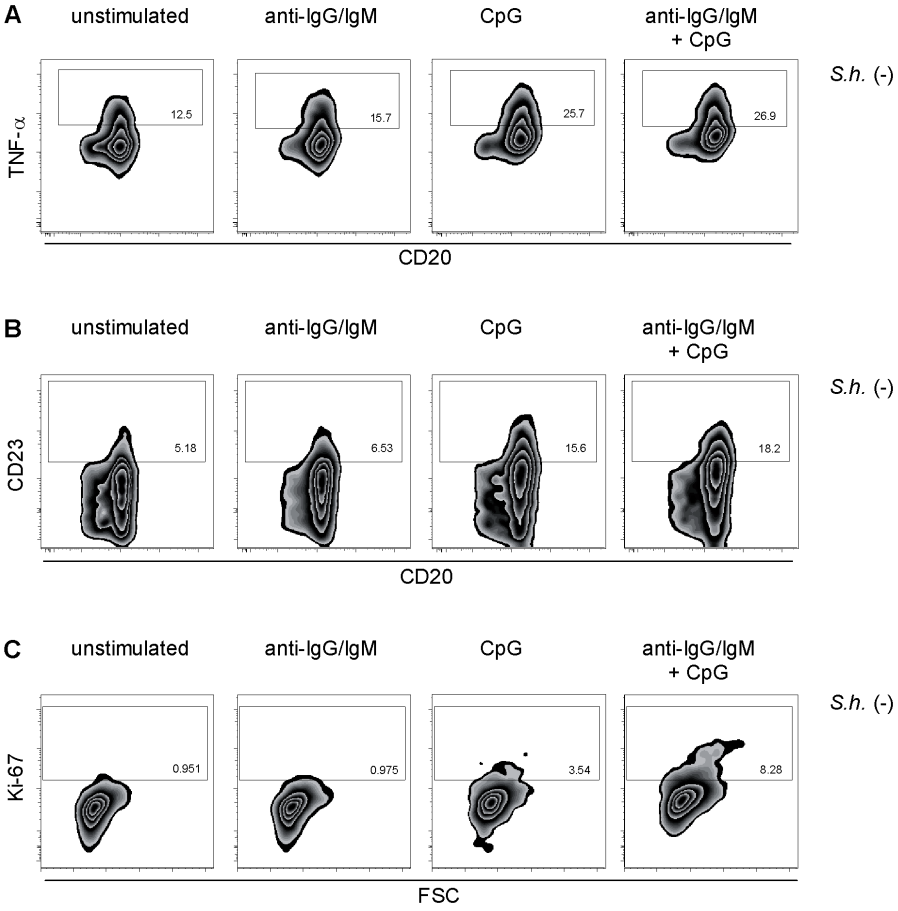
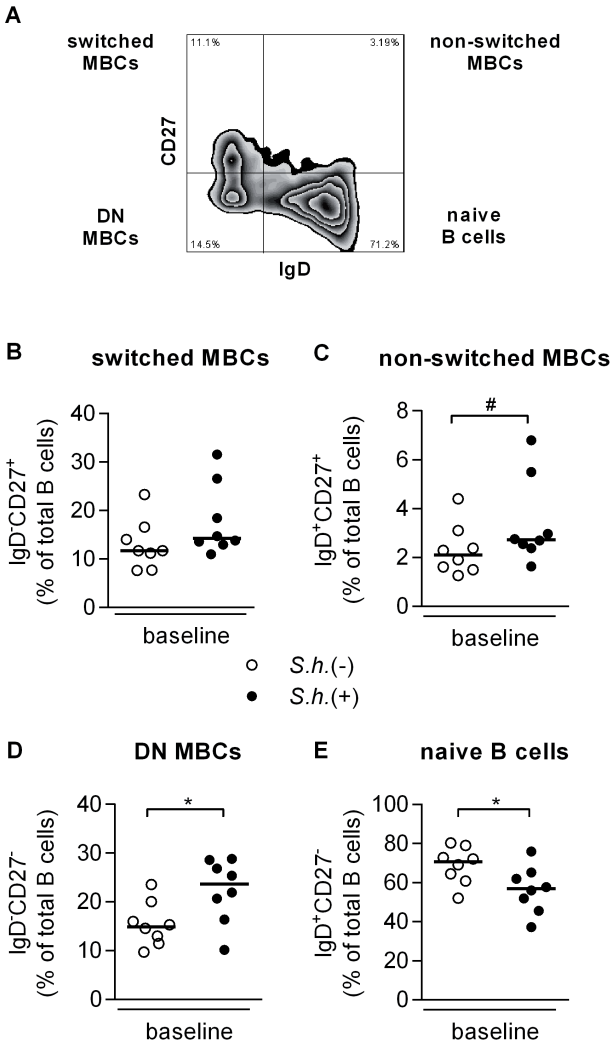
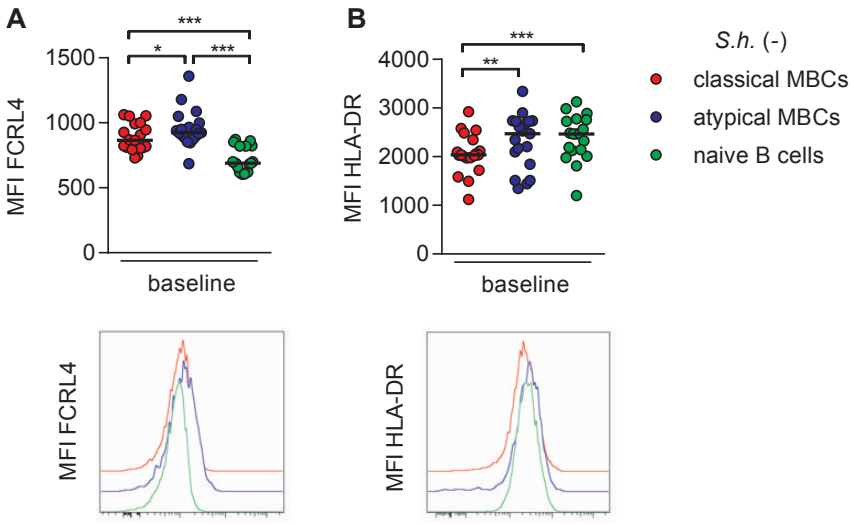
Supplementary material



Supplementary Figure 1. Gating strategy for B cell inflammatory cytokine response, activation and proliferation.
Total peripheral blood B cells were cultured with anti-IgG/IgM (2.5 µg/ml), CpG (5 µg/ml) or anti-IgG/IgM plus CpG for two days, restimulated with PMA/Ionomycin/LPS and BrefA and fixed. Levels of intracellular TNF-α (A), CD23 expression (B) and intracellular Ki-67 (C) were gated according to the gating strategy depicted in this figure (representative *S. haematobium*-uninfected child).



Supplementary Figure 2. MBC analysis.
PBMC were fixed and stained with B cell phenotyping markers (CD19, CD27 and IgD) and analyzed for B cell subsets by flow cytometry. B cell subset analysis was performed as shown in (A) (representative *S. haematobium*-uninfected child). Proportion of CD19-gated cells that were $CD27^{+}IgD^{-}$ (B, switched MBC), $CD27^{+}IgD^{+}$ (C, non-switched MBC), $CD27^{-}IgD^{-}$ (D, double negative MBC), and $CD27^{-}IgD^{+}$ (E, naïve B cells) were determined for *S. haematobium*-infected and uninfected children at baseline. (B, C, D, E) Horizontal bars represent median. Number of donors in each group: baseline *S.h.* –ve n = 8 and *S.h.* +ve n = 8.



Supplementary Figure 3. Expression of FCRL4 and HLA-DR on B cell subpopulations.
PBMC were fixed and stained with B cell subset markers (CD19, CD21 and CD27) and measured for FCRL4 (A) and HLA-DR (B) expression in *S. haematobium*-uninfected children by flow cytometry. Histograms of MFI underneath are from a representative child. Horizontal bars represent median. Number of donors: baseline *S.h.* –ve n = 19.

8

General Discussion.

A successful response against invading pathogens results from a complex interplay between the many diverse cell types of the immune system. Following pathogen exposure a non-specific innate immune response is orchestrated by neutrophils, macrophages, NK cells and eosinophils which act as the first line of defense against invading organisms [1]. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which recognize pathogen-associated molecular patterns (PAMPs) play a key role at this stage [2]. Next an antigen-specific adaptive immune response, which may take days to develop, is mounted. Here antigen presenting cells (APCs) play an important role in linking innate and adaptive immune responses and direct the initiation and polarization of T and B cell activities. T cells differentiate into various effector cells depending on the type of infection, while B cells undergo differentiation and most will become antibody-producing plasma cells [1]. The Th1 (IFN-γ producers) / Th2 (IL-4, IL-5 an IL-13 producers) paradigm of T helper subsets has recently been expanded to include the immunosuppressive IL-10/TGF-β-producing $CD4^+CD25^+FOXP3^+$ T regulatory T cells (Tregs) and the pro-inflammatory IL-17-producing T cells (Th17) [3]. Interaction between B cells and T cells is also important for adaptive immunity; $CD4^+$ T cells provide help to B cells [4] and new data has shown that B cells also act as modulators of T cell responses via their effector and regulatory functions [5].

The elucidation of the various interactions and activities between these different components is essential to fully understand the mechanisms during human schistosome infection which will then in turn provide better foundation for the design and implementation of strategies to manage, prevent and eradicate this disease. Knowledge gained here could also have important bearings on other helminth infections.

In this thesis we have investigated cross-sectionally and longitudinally the effect of schistosomes on several aspects of the immune system, including innate, adaptive and regulatory responses, in a group of schoolchildren in an area in Gabon where *S. haematobium* is endemic.

Innate Immune Responses

In Chapters 2, 3 and 4 we have investigated innate immune responses directed against TLR and CLR ligands in *S. haematobium*-infected children and in uninfected controls by measuring cytokine responses against these ligands in either whole blood or PBMC cultures. We have found that while in PBMC cultures differences were observed to the TLR2/1 ligand Pam3, with higher levels of the pro-inflammatory cytokine TNF in *S. haematobium* infected children, this difference was no longer detectable when the same response was analyzed in whole blood cultures. The initial finding of an increased pro-inflammatory innate immune response in PBMC cultures to TLR stimulation (Chapter 3) was rather intriguing as schistosome infection has, for the most part, been characterized in terms of strong antigen-specific Th2 and regulatory responses resulting in immune hypo-responsiveness [6–8]. These findings challenged the predominant view of general immune suppression induced by the parasite and showed that hypo-responsiveness did not extend to innate immune responses in the context of single TLR ligation. However, when whole blood cultures were stimulated with the TLR ligands, we did not see a higher pro-inflammatory response in *S. haematobium* infected subjects (Chapters 2 and

4). The differences between whole blood and PBMC cultures may result from differences in cell composition. Whole blood assays reflect an environment in which the different cell types in their *in vivo* ratios are present and contain cells such as erythrocytes, and granulocytes as well as plasma which may influence or contribute to the cytokine response measured [9]. Neutrophils and eosinophils in particular are known to produce many Th1/Th2/pro-inflammatory cytokines which may alter the cytokine milieu resulting in differences between total cytokines measured in whole blood and PBMC cultures [10,11]. Moreover, in whole blood assays the number of cells cultured is not known nor is it controlled for, and thus changes in the number of cytokine producing cells may be responsible for the differences observed rather than the ability of cells to produce cytokines. In contrast, PBMC cultures use the same number of cells for each individual thereby giving a more controlled measure of the functionality of the studied cells. Importantly, as shown in Chapter 4, the presence or absence of co-infections such as malaria can contribute to innate immune responsiveness; this infection can change with the season or the specific area of a study and thereby influence responses measured.

In addition to TLR responses we also investigated two other classes of PRRs, the C-type lectin receptors (CLRs) (Chapters 2 and 4) and nucleotide-binding oligomerisation domain-like receptors (NLRs) (Chapter 2). Responses of these receptors cannot be studied on their own as most do not contain signaling domains and therefore do not lead to cytokine production. It has been proposed that innate immune responses can be fine-tuned via interaction between distinct PRRs [12,13]. An elegant way of studying their function is to look at the ability of these receptors to enhance or diminish TLR responses. While we found significant interactions between the different classes of PRRs, both synergistic and inhibitory, we did not find any differences in these responses between *S. haematobium*-infected and uninfected children. Nonetheless there is increasing evidence that schistosome antigen recognition by host C-type lectins plays an important role in shaping the immune response against infection [14]. Schistosomes express various carbohydrates containing glycoproteins on their surface and release glycan-rich E/S products that have been shown to bind to various CLRs, including DC-SIGN, MR, MGL [15] and Dectin-2 [16]. Furthermore, increased expression of DC-SIGN on DCs was recently shown to be required for Th17 cell differentiation in response to schistosome eggs and the development of immunopathology in a mouse model of *S. mansoni* infection [17]. It would be of interest to study responses to CLRs in PBMC cultures, or in well defined, specific cell subsets, incorporating flow cytometry to measure receptor expression on cells before and after stimulation.

In Chapter 2 we compared responses between the different groups of schoolchildren from Gabon and an age-matched group of European children from the Netherlands to help us understand how the innate immune response can be affected by large geographical and environmental influences. We did not observe any differences in the interaction between the different classes of PRRs between these groups; however we did find a significant difference in TLR responsiveness. Gabonese children had a lower pro-inflammatory response to poly(I:C) (TLR3 ligand), but a higher pro-inflammatory response to FSL-1 (TLR2/6 ligand), Pam3 (TLR2/1 ligand) and LPS (TLR4 ligand) compared to Dutch children. Anti-inflammatory responses to Pam3 were also higher in Gabonese children. Differences in these responses may result from differences in expression [18,19], signaling [20] or genetic polymorphisms [21] in TLRs or in

molecules involved downstream but it is also possible that environmental exposures shape the contrasting innate immune responses. Environmental exposures to viruses, bacteria and parasites may have played a role by resulting in an imprinted 'memory' which has recently been termed 'trained innate immunity' [22]. Differences between being born and raised in a high-income (Netherlands) versus a low-income (Gabon) country, dietary habits or vaccination schedules may have further played an important role.

Adaptive Immune Responses

In Chapters 3 and 4 we investigated adaptive immune responses directed against schistosome soluble egg (SEA) and adult worm antigens (AWA) and in Chapter 5 we further extended this analysis to the vaccine-antigen Bacillus Calmette–Guérin (BCG). In Chapter 7 we carried out an extensive phenotypic investigation of the memory B cells subsets.

We found that *S. haematobium*-infected children had significantly higher levels of IL-10 in response to SEA, and IL-5, IL-10 and IL-2 in response to AWA compared to the uninfected controls. Interestingly, IL-10 levels were increased in infected children irrespective of whether this cytokine was measured in whole blood (Chapter 4) or in PBMC cultures (Chapter 3) demonstrating the reproducibility of the increase in this anti-inflammatory regulatory response. Higher IL-10 levels are in line with previous studies in *S. haematobium* [23,24] and filarial infection [25]. Anti-schistosome treatment with praziquantel resulted in the increase of the levels of SEA and AWA specific IL-5 and IL-10, SEA specific TNF, and AWA specific IL-2 (Chapter 4). In Chapter 5 we used Principle Component Analysis (PCA) to describe global changes in cytokine responses following schistosome treatment in response to not only SEA and AWA stimulation but also to a third-party antigen BCG. PCA allows the reduction of large datasets into summary variables termed principal components with each principal component representing variables that share a high level of correlation [26]. In the current study we identified two distinct principal components: principle component 1 (PC1) which reflects regulatory and Th2-polarized cytokine responses due to its positive loading with IL-5, IL-10 and IL-13 responses; and principle component 2 (PC2) which reflects pro-inflammatory and Th1-polarized cytokine responses due to its positive loading with IFN-γ, IL-17 and TNF. We saw a significant increase in both PC1 and PC2 following treatment compared to baseline values. These results are in line with a number of short-term (weeks) as well as long-term (months) treatment studies, which likewise show enhanced antigen specific responses following removal of infection [27–31]. An increase in all four types of immune responses i.e. Th1, Th2, regulatory and pro-inflammatory, suggests that treatment results in the removal of general schistosome-mediated immunosuppression of adaptive responses, but may also in part be due to the release of previously cryptic antigens from the dying parasites resulting in boosting of the recall response [28,32]. Indeed repeated anthelminthic treatment and therefore by extension repeated exposure to antigen has been shown to result in greater cytokine production than single treatment [33]. Nonetheless strategies which would further disentangle immunosuppression and regulatory responses, from enhanced responses due to antigen release are warranted. The role of regulatory responses in schistosomes-induced hypo-responsiveness is discussed in detail in the next section.

B cells are key effector cells in the adaptive humoral immune response during schistosome

infection [34–36]. Multiple phenotypically distinct memory B cell (MBC) subsets have been characterized in humans [37–39]. The study presented in Chapter 7 of this thesis investigated the frequency of these subsets based on differential expression of CD27, CD21 and IgD. Frequencies of switched, double negative and activated MBCs, as well as a trend toward a higher percentage of atypical MBCs was observed in schistosome-infected children. A concomitant decrease of naïve B cells was also observed. These profiles were restored to those observed in uninfected children following treatment. It is of particular interest that double negative MBCs as well as the atypical MBCs were increased during infection as these two subsets have been linked to hypo-responsiveness and an exhausted phenotype in HIV- [39], in malaria-infected individuals [40,41], and in patients with systemic lupus erythematosus [42,43], a chronic autoimmune disease. Atypical MBCs have a decreased ability to differentiate into antibody secreting cells resulting in reduced pathogen-specific antibody responses in infected individuals. The capacity of these cells to produce schistosome-specific antibodies or the extent of their exhausted phenotype is currently not known. However, an increase in IgG^+ double negative ($CD27^-IgD^-$) MBCs was observed in *S. haematobium* infected children which reflected the increase in total serum IgG4 levels. Following praziquantel treatment there was a concomitant decrease in the frequency of the DN MBCs and serum levels of IgG4, suggesting that the increase in IgG^+ DN MBCs may be predominantly due to an increase in IgG4-expressing B cells during infection. As IgG4 is associated with susceptibility and IgE with resistance to schistosome infection, it would be of interest to study these isotypes on the different memory B cell populations in exposed but resistant individuals.

Regulatory Responses

Recent studies have emphasized the significant role of the regulatory networks in the immune suppression induced by parasitic infections [6]. The role of regulatory T cells (Tregs) in particular has shown the multifaceted nature of this immune response. Accumulating evidence has shown that parasitic helminths induce Treg expansion and/or activity. These cells produce down-modulatory cytokines such as IL-10 and TGF-β that lead to a dampened immune response [44,45]. These are in line with studies in murine models where the abrogation of Treg activity leads to recovery from chronic parasite infection by restoring immune function [46–48]

In Chapter 4, in addition to measuring cytokine responses to schistosome-specific antigens we concurrently evaluated the frequency of $CD4^+CD25^+FOXP3^+$ T cells. We found that the frequency of $CD4^+CD25^+FOXP3^+$ T cells was significantly increased in *S. haematobium*-infected schoolchildren and reduced to 'normal' levels after praziquantel treatment. The differences between infection groups in the frequency of $CD4^+CD25^+FOXP3^+$ T and the change in these over time showed an inverse pattern to antigen-specific cytokine responses. Using a linear mixed-effects model to assess the longitudinal association between $CD4^+CD25^+FOXP3^+$ T cell levels and cytokine responses to schistosomal antigens we showed that the decrease in the frequency of $CD4^+CD25^+FOXP3^+$ T cells over time following treatment is inversely associated with an increase in IL-5 and IL-10 cytokine production. Alongside the decrease in $CD4^+CD25^+FOXP3^+$ T cells and the increase in antigen-specific cytokine responses we also observed an increase in the effector memory (T_{EM}) T cells in the infected children following

treatment suggesting that hypo-responsiveness may also in part be linked to the memory T cell pool. However, as we did not have sufficient data across all time points for T_{EM} frequencies we were not able to assess the longitudinal association between T_{EM} and cytokine levels and $CD4^+CD25^+FOXP3^+$ T cell frequencies. To further assess the functional contribution of $CD4^+CD25^+FOXP3^+$ T cells to *in vitro* immune responses, we performed magnetic depletion of $CD25^{hi}$ cells and analysed cytokine responses before and after $CD4^+CD25^+FOXP3^+$ T cell depletion at both pre- and post-treatment in Chapter 5. As in Chapter 4 we found a significant decrease in $CD4^+CD25^+FOXP3^+$ T cells following treatment. Similar to evaluating adaptive cytokine responses in Chapter 4, here we also evaluated the effect of $CD4^+CD25^+FOXP3^+$ T cell depletion on principal component 1 (IL-5, IL-10 and IL-13) and principal component 2 (IFN-γ, IL-17 and TNF). We found $CD4^+CD25^+FOXP3^+$ T cell depletion resulted in increased values of both PC1 and PC2 in infected individuals. Although levels of $CD4^+CD25^+FOXP3^+$ T cells were decreased following treatment their suppressive capacity was intact: the depletion of the regulatory T cells at post treatment also led to increase in PC1 and PC2. We also evaluated the effect of $CD4^+CD25^+FOXP3^+$ T cell depletion on cell proliferation. Interestingly, while $CD4^+CD25^+FOXP3^+$ T cell depletion resulted in similar increase in cytokine production at both pre- and post-treatment, proliferative responses were for the most part only significantly affected by $CD4^+CD25^+FOXP3^+$ T cell depletion in infected individuals at pre-treatment. Following removal of infection $CD4^+CD25^+FOXP3^+$ T cell depletion no longer suppressed cell proliferation. This suggests that while a reduction in $CD4^+CD25^+FOXP3^+$ T cell numbers is sufficient to abrogate the suppressive qualities of $CD4^+CD25^+FOXP3^+$ T cells on proliferation, the functional changes induced in $CD4^+CD25^+FOXP3^+$ T cells by schistosome infection still persist in terms of their ability to influence the production of effector cytokines 6 weeks after treatment. Interestingly, an IL-10 producing $CD8^+CD25^+FOXP3^+$ T cell population has also been recently described [49–51] and as $CD25^+$ cell depletion will, in addition to depleting $CD4^+CD25^+FOXP3^+$ T cells, also deplete the $CD8^+CD25^+FOXP3^+$ T cell population, future studies are needed to re-assess the relative contributions of these subsets. Moreover as FOXP3 expression may be transiently up-regulated on activated $CD4^+$ T cells [52], future studies will need to include more extensive panels of markers associated with suppressive T cell functions.

Although much research has focused on the role of regulatory T cells it is likely that other immune cells are also involved. While predominantly characterized as being involved in humoral immunity through the production of antibodies, B lymphocytes possess multiple additional functions, including production of cytokines, for example IL-10 and TGF-β, and the ability to function as APCs through the expression of MHC class II molecules which are involved in presentation of antigens to T cells [53–55]. In addition, these cells express a variety of PRRs, in particular TLRs, which might be involved in the amplification and possible polarization of the signals given to T cells that are being activated by B cells [56–59]. They have been shown to be involved in immune tolerance and suppression of disease including inflammatory bowel disease, rheumatoid arthritis, experimental autoimmune encephalomyelitis and multiple sclerosis [55]. More importantly they have also been shown to be involved in the induction of immune regulation during parasitic infections, such as T*oxoplasma gondii, Heligmosomides polygrus* [60] and schistosomiasis. For example, μMT mice die rapidly during the course of

S.mansoni infection compared to wild-type mice [61]. Furthermore a number of regulatory B cell subsets (Bregs) have been characterised in humans [62–64] and in Chapter 6 we assessed the frequency of these subsets. We found no differences in the levels of $CD24^{hi}CD38^{hi}$ or $CD24^{hi}CD27^{+}$, but we did observe a significant increase in the frequency of $CD1d^{hi}(CD5^{+})$ Bregs in schistosome infected children. The increase in $CD1d^{hi}(CD5^{+})$ Bregs was accompanied by an increase in IL-10-producing B cells in the total B cell population. In particular $CD1d^{hi}$ B cells from infected children produced more IL-10 as compared to uninfected children. Both the frequency of $CD1d^{hi}(CD5^{+})$ Bregs and total IL-10 levels decreased following treatment to levels comparable to the uninfected children. Schistosome-specific IL-10 in $CD1d^{hi}(CD5^{+})$ Bregs however were not down-regulated following treatment suggesting that a small population of schistosomes-specific B cells that more readily produces IL-10 in response to SEA persists in infected children.

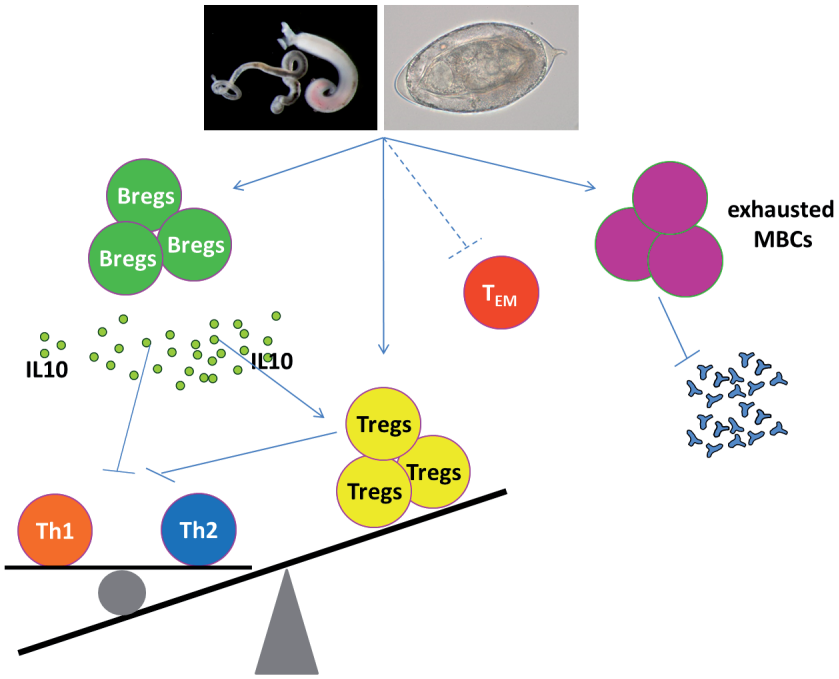


Figure 1. A schematic representation of innate, adaptive and regulatory immune responses in human schistosomiasis in Gabon.

S. haematobium infection induces increased frequencies of regulatory B (Breg) and T (Treg) cell subsets which are associated with increased levels of IL-10 and hypo-responsiveness, possibly in effector memory T cells (T_{EM}). In addition, exhausted B cell are also increased. Praziquantel treatment results in the reduction of regulatory and exhausted subsets, an increase in effector T cells and alleviation of suppressed antigen immune responses.

Immunosupression as a result of anergy

An alternative or perhaps a concurrent explanation for suppression of antigen-specific cytokine responses by regulatory cells during schistosome infection is an intrinsic unresponsive or hypo-responsive state of the T cells. T cell anergy has been described during chronic helminth infection in both mice and humans, where $CD4^+$ Th2 cells develop an intrinsically unresponsive functional state [45]. The hypo-responsiveness in Th2 cells has been shown to be dependent on the up-regulation of the expression of the E3 ubiquitin ligase GRAIL (gene related to anergy in lymphocytes). Removal of Th2 cells from antigen exposure results in the down-regulation of GRAIL and a dramatic restoration of function [65]. Mouse studies have also shown a role for a number of inhibitory receptors, including PD-1 and TIM-1, and expression of PD-1 ligands PD-L1 and PD-L2 by macrophages that inhibit T cell immunity in *S. mansoni* infection [66–68]. Future studies are needed to address the role of these molecules in human infections.

Spill-over suppression

The strong immunoregulatory network that induces immunosuppression during the course of schistosomiasis, and also during other helminth infections, can be both detrimental as well as beneficial. Spill-over suppression to third-party antigens may lead to impaired responses to infections, cancers or vaccines. Helminth infections induce a Th2 bias, while a strong Th1 response is desirable during vaccination. Impaired Th1 responses to Bacille Calmette- Guérin (BCG), to tetanus toxoid, and to influenza virus have been seen in helminth infected individuals [69–71]. Another detrimental effect to be considered is that helminth infections may exert a negative role on cancer incidence or progression [72]. It is thought that a 'healthy' immune system can naturally control spontaneously arising tumours, and it has been shown that immune deficiencies can predispose to carcinogenesis [73], therefore an immunosuppressive environment induced by helminth infection may prevent the host from mounting an effective response against cancers or impair responses to anti-cancer therapeutics. The negative effects of helminths may also extend to responses to concurrent infections [74–76]. Protective immune responses against *P. falciparum* are associated with a Th1 response which leads to production of protective IgG1 and IgG3 antibodies [77,78]; Th1 responses can be down-regulated during chronic schistosome infection where responses are skewed towards Th2 [79]. With respect to clinical outcome of malaria, which is thought to result from strong pro-inflammatory response, there are studies that have shown a protective effect of helminths on malaria [80,81]. This is thought to be as a result of the ability of helminths to induce regulatory T cells which by down-regulating strong inflammatory responses could prevent the incidence of clinical malaria.

On the other hand spill-over suppression to third party antigens might be beneficial against excessive inflammatory responses observed in allergies, asthma, autoimmune diseases and even cardiovascular diseases or metabolic disorders. A large number of epidemiological studies on the prevalence of allergies in helminth infected individuals have shown a negative association between helminth infections and allergies (in particular skin reactivity to allergens) [82,83]. Various helminth species have also been shown to limit inflammatory activity in a variety of diseases including inflammatory bowel disease (IBD), multiple sclerosis (MS), type 1 diabetes, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and cardiovascular

disease (CVD) [84,85] .The protective role during helminth infections has for the most part been linked with the induction of the regulatory network [86]. A number of clinical trials with either *Trichuris suis* (the pig whipworm) eggs or *Necator americanus* (human hookworm) live worms have been conducted and several are currently underway investigating the effects on MS, IBD, allergic rhinitis, celiac disease and even autism [86]. Ideally however, specific helminth-derived molecules would be needed for therapeutic application to remove the negative aspects of helminth infections in these treatment strategies.

Concluding remarks and future perspectives

The studies described in this thesis have demonstrated the interconnectedness between the various arms of the immune response mounted against and induced by *S. haematobium* infection. By showing that regulatory T cells are linked to effector responses in schistosomiasis and that schistosomes can induce regulatory B cells, the scene is set for future studies to determine antigen specificity of these cells as well as ways to control their activity.

As regulatory responses have been shown to be not only important in chronic infectious disease, but also in chronic inflammatory diseases the knowledge gained here may be of substantial value to the health of those living in both low- to middle-income countries as well as high-income countries. Specifically, a number of important issues need to be considered:

- Regulatory responses induced by helminth infections can affect immune responses to vaccines. Therefore helminth status should be an important consideration for vaccination programs and trials; deworming is needed for optimal vaccine efficacy.

- Targeted drug therapy and population-based treatment programs are currently advocated by multiple agencies, including the WHO, as major components of schistosomiasis control strategies. Mass drug administration programs are also in place to treat other helminth infections. However, as deworming might be positively associated with allergy and inflammatory diseases it is imperative that follow-up studies on immunological parameters in individuals from endemic settings are conducted to monitor the effects on the development of inflammatory conditions.

- Extensive characterisation of immune response during infection will furthermore pave the way for more successful vaccine development against schistosomiasis which is sorely needed as re-infection rates are extremely high in endemic settings and repeated drug treatment may lead to drug-resistant schistosomiasis.

The use of novel high-dimensional technologies such as trancriptomics, metabolomics and microbiomics will further improve our understanding and give a more complete picture of the effect that *S. haematobium* has on the human host and how this can be exploited.

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English Summary
Nederlandse Samenvatting
Curriculum Vitae
List of publications
Acknowledgements

English Summary

Parasitic infections have been a human companion for millennia and currently a quarter of the world's population are infected with helminth worms. The long co-existence of worms and humans has profoundly influenced the human immune response. A healthy immune response will have a fine balance between effector mechanism directed against invading pathogens and regulatory mechanisms which keep the effector mechanisms in check and protect the body from tissue damage and inflammation. As our immune system has evolved to function in pathogen-rich environments, recent improvements in healthcare and hygiene may contribute to increased incidence of allergy, autoimmune disorders, cardiovascular diseases and other inflammatory conditions observed in Westernized countries today. It has been proposed that the decrease in infectious diseases is directly related to the increase in these disorders due to insufficient education of the immune system and an underdeveloped regulatory response. Chronic helminth infections, including schistosomiasis, have been shown to induce regulatory mechanisms, and the protective effect of helminth infections is very likely due to the down-regulation of the immune response by the parasite to ensure its own survival, which extends to bystander antigens i.e. allergens or self-antigens. However, down- regulation of the immune response, while protective against innocuous challenges, may also adversely affect immune responses against other pathogens, the efficacy of drug treatments or vaccine responses.

The mechanisms and consequences of immunomodulation induced by helminth infections are therefore of tremendous interest, as a better understanding of these will contribute towards new possibilities for control of infection and pathology and therapeutic applications in the treatment or even prevention of hyper-inflammatory disorders.

The work presented in this thesis is an investigation of the immune responses induced by chronic schistosomiasis in Gabonese schoolchildren. By investigating concurrently various aspects of the immune response we are able to gain a more in-depth understanding of the dynamic changes brought about by infection.

In **Chapter 1**, the background and context of the research is described, the study population and the area are introduced and the study objectives are outlined.

In **Chapter 2**, we compare innate immune responses to several classes of pathogen recognition receptors (PRRs) between semi-urban and rural African schoolchildren and European schoolchildren using identical reagents and experimental protocols in order to assess whether innate responses are affected by environmental factors. We show that while differences in response to Toll-like receptors (TLRs) stimulation are present, no differences to C-type lectin receptors (CLRs) or nucleotide-binding oligomerisation domain-like receptors (NLRs) were detected. Possible explanations for the differences in cytokine production observed in response to TLR ligands including variations in TLR expression levels, differences in downstream signalling pathways or differential exposure to viruses, bacteria and parasites are discussed.

Chapter 3 addresses differences in innate or non-specific and adaptive or specific immune responses in peripheral blood mononuclear cells (PBMCs) between *S. haematobium*-infected schoolchildren and uninfected controls. We find that innate immune responses are pro-inflammatory and that adaptive immune responses are anti-inflammatory. Anti-inflammatory

responses play an important role in adaptive immune hypo-responsiveness, a hallmark of chronic schistosomiasis, and we show that immune hypo-responsiveness induced by *S. haematobium* infection does not extend to innate immune responses.

In **Chapter 4**, we compare whole blood immune responses of *S. haematobium*-infected children before and after treatment with praziquantel and in parallel include uninfected children as controls. We find that innate immune responses do not differ between infection groups. The difference in innate responses observed between Chapters 3 and 4 may be explained by differences in cell composition in whole blood (in Chapter 4) versus PBMC (in Chapter 3). With respect to adaptive immune responses, infected children recognized schistosomal antigens, whereas uninfected children did not. Moreover, we show that antigen-specific responses that are seen in infected children are significantly increased following removal of infection. We provide evidence that regulatory T cells ($CD4^+CD25^+FoxP3^+$) which were higher in infected children, decreased following anti-schistosome treatment and this decrease is associated with an increase in adaptive immune responses. These results for the first time provide evidence for the association between schistosome infection, regulatory T cells and antigen-specific immune hypo-responsiveness in humans.

In **Chapter 5**, we further expand our analysis of regulatory T cells and investigate the role of $CD4^+CD25^+FoxP3^+$ regulatory T cells on cell proliferation and cytokine production in response to antigens in PBMCs before and after praziquantel treatment. We use principal component analysis (PCA), a data reduction approach, to study cytokine responses globally. We show that schistosome infection is associated with increased frequencies of Treg cells, which decrease following treatment. The reduction in Treg frequencies was accompanied by an increase in cytokine responses following treatment. Treg depletion studies confirm the inhibitory nature of these cells as their depletion resulted in increased cytokine production both at pre-treatment and post-treatment. Proliferative responses for the most part were not as significantly affected as cytokine responses.

We next address the role of B cells in the course of schistsome infection. In **Chapter 6**, we show novel data that schistosomiasis induces regulatory B cell (Breg) cells which like regulatory T cells are negative regulators of immune responses. We show that Gabonese schoolchildren have an elevated population of $CD1d^{hi}$ B cells and that these cells produce elevated levels of IL-10. Importantly, after anti-schistosome treatment the frequency of these cells is reduced to levels comparable to the uninfected children.

In **Chapter 7**, we show that in addition to altered frequencies of Bregs, the frequency of memory B cell (MBC) subsets is increased in infected schoolchildren. At the functional level, these cells, while more activated, have reduced proliferation and pro-inflammatory cytokine production. Treatment resulted for the most part in restoration of function. Results in this chapter show that *S.haematobium* infection leads to significant changes in the B cell compartment both at the phenotypic and functional level.

Finally, in **Chapter 8**, the findings of this thesis are summarized. The interaction between innate, adaptive and regulatory responses is discussed and in particular emphasis is placed on regulatory responses. As regulatory responses have been shown to be not only important in chronic infectious disease, but also in chronic inflammatory diseases, the knowledge gained

here may be of substantial value to the health of those living with infection and those currently affected by inflammatory conditions.

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Nederlandse Samenvatting

Mensen dragen al millennia lang parasieten met zich mee en momenteel is een kwart van de wereldbevolking geïnfecteerd met wormen. Aangezien mensen in de loop van de evolutie voortdurend bloot zijn gesteld aan een rijke microbiële omgeving, ligt het in de lijn der verwachting dat de relatie van mensen tot 'hun' wormen gezien kan worden als één met commensalen en het afweersysteem ook ontwikkeld is om optimaal te functioneren in de voortdurende aanwezigheid van deze commensalen. Een gezonde afweerreactie wordt gekenmerkt door een balans tussen ontstekingsreacties gericht tegen binnendringende ziekteverwekkers, genaamd pathogenen, en tolerantieprocessen die deze ontstekingsreacties in toom moeten houden wanneer de pathogenen opgeruimd zijn. Deze tolerantiemechanismen beschermen het lichaam tegen weefselschade veroorzaakt door de ontstekingsreacties. In de afgelopen decennia is de gezondheidzorg en hygiëne in de westerse wereld enorm verbeterd, maar tegelijkertijd nam het aantal gevallen van ontstekingsziekten zoals allergieën, auto-immuunziekten en hart- en vaatziekten enorm toe. Verondersteld wordt dat een direct verband bestaat tussen de toename in deze ontstekingsziekten en de afname in infectieziekten. Aangezien ons afweersysteem is geëvolueerd om te functioneren in een pathogeenrijke omgeving, vermoedt men dat door de afname van infectieziekten het afweersysteem, en dan met name de aanmaak van tolerantieprocessen, onvoldoende wordt getraind. Daarentegen komen deze typische westerse ontstekingsziekten nog weinig voor in ontwikkelingslanden, maar komen er nog wel veel parasitaire infecties voor. Het is aangetoond dat chronische worminfecties, waaronder schistosomiasis, tolerantieprocessen kunnen opwekken. Het beschermende effect van worminfecties wordt vermoedelijk veroorzaakt door onderdrukking van ontstekingsreacties, waarmee de parasiet zijn eigen overleving veilig stelt. Deze tolerantieprocessen zouden ook neveneffecten veroorzaken, zoals een verminderde ontstekingsreactie op onschuldige allergenen zoals huisstofmijt en graspollen en lichaamseigen stoffen. Hoewel deze tolerantieprocessen op een positieve manier beschermen tegen overbodige reacties op onschuldige stoffen, zouden ook ontstekingsreacties tegen andere pathogenen en de effectiviteit van medicijnen en vaccins op een nadelige manier beïnvloed kunnen worden.

Onderzoek naar de mechanismen en gevolgen van de opgewekte ontstekingsreacties veroorzaakt door worminfecties zijn van groot belang, aangezien een beter begrip ervan kan bijdragen aan nieuwe mogelijkheden om infecties en weefselschade te beheersen en om zo typische westerse ontstekingsziekten te behandelen of zelfs te voorkomen.

Dit proefschrift beschrijft onderzoek naar afweerreacties tegen chronische schistosomiasis in Gabonese schoolkinderen. Door gelijktijdig verschillende aspecten van de afweerreacties te onderzoeken, zijn we in staat de dynamische veranderingen veroorzaakt door de infecties, beter te begrijpen.

In **hoofdstuk 1** wordt de achtergrond en context van het onderzoek beschreven, worden de studiepopulatie en het studiegebied geïntroduceerd en worden de studiedoelstellingen uiteengezet.

In **hoofdstuk 2** vergelijken we de aangeboren of aspecifieke afweerreacties in het bloed van semi-stedelijke en landelijke Afrikaanse schoolkinderen en Europese schoolkinderen door het

gebruik van identieke reagentia en experimentele protocollen. De stimulatie van verschillende soorten receptoren die pathogenen herkennen, zoals Toll-'like'-receptoren (TLR), C-type-lectine-receptoren (CLR) en nucleotide-bindende-oligomerisatie-domein-'like'-receptoren (NLR), werd onderzocht zodat kon worden vastgesteld of aangeboren afweerreacties beïnvloed werden door omgevingsfactoren. We tonen aan dat, hoewel er verschillen waren in reacties op stimulatie van TLR, er geen verschillen werden gedetecteerd bij CLR of NLR receptoren. In dit hoofdstuk worden mogelijke verklaringen besproken voor de verschillen in productie van cytokines (signaalstoffen) die zijn waargenomen in reactie op TLR stimulaties, waaronder mogelijke verschillen in de genexpressie van de TLR receptoren, verschillen in onderliggende signaleringsroutes of verschillende blootstelling aan virussen, bacteriën en parasieten.

Hoofdstuk 3 richt zich op verschillen in aangeboren (of aspecifieke) en verworven (of adaptieve) ontstekingsreacties in witte bloedcellen uit veneus bloed tussen *S. haematobium*-geïnfecteerde en ongeïnfecteerde schoolkinderen. We hebben gevonden dat aangeboren afweerreacties ontstekingsbevorderend werken en verworven reacties ontstekingsremmend. We tonen aan dat de ontstekingsremmende reacties, ofwel tolerantieprocessen, die een belangrijke rol spelen in immuunhyporesponsiviteit van adaptieve of verworven afweerreacties bij chronische schistosomiasis, zich niet uitstrekken tot aangeboren afweerreacties.

In **hoofdstuk 4** vergelijken we afweerreacties in volbloedkweken van *S. haematobium*-geïnfecteerde kinderen voor en na behandeling met praziquantel en in parallel met ongeïnfecteerde kinderen als controles. We tonen aan dat aangeboren afweerreacties niet verschillen binnen de infectiegroepen. Het verschil in aangeboren afweerreacties tussen hoofdstuk 3 en 4 zou verklaard kunnen worden door verschillen in celsamenstelling in het bloed (in hoofdstuk 4) en in de geïsoleerde witte bloedcellen (in hoofdstuk 3). In het geval van de verworven afweerreacties herkenden de cellen van geïnfecteerde kinderen de moleculen van schistosoom-wormen, terwijl de cellen van niet-geïnfecteerde kinderen dat niet deden. Bovendien hebben we aangetoond dat de reacties gericht tegen wormmoleculen in geïnfecteerde kinderen significant waren toegenomen na behandeling van infectie. We vonden een hoger percentage van regulatoire T cellen (een groep van tolerantie-bevorderende witte bloedcellen, gekarakteriseerd door de expressie van markers $CD4^+CD25^+FOXP3^+$), in geïnfecteerde kinderen, wat vervolgens weer afnam na een behandeling tegen schistsomiasis. Deze afname was geassocieerd met een toename in verworven afweerreacties. Deze resultaten bieden voor het eerst bewijs voor de associatie tussen schistosoma-infecties, regulatoire T cellen en worm-specifieke immuunhyporesponsiviteit in mensen.

In **hoofdstuk 5** breiden we onze analyse van regulatoire T cellen verder uit en onderzoeken we de rol van $CD4^+CD25^+FOXP3^+$ regulatoire T cellen in celdeling en cytokineproductie in reactie op wormmoleculen in witte bloedcellen voor en na praziquantel behandeling. We gebruiken principale-componentenanalyse (PCA), een datareductie-benadering, om de verschillende cytokines in het algemeen te bestuderen. We hebben aangetoond dat schistosoom-infectie is geassocieerd met hogere frequenties regulatoire T cellen, die daalden na behandeling. De afname in regulatoire-T–cel-frequenties ging gepaard met een toename in cytokineproductie tijdens behandeling. Studies waarin de regulatoire T cellen werden verwijderd, bevestigden het remmende karakter van deze cellen, aangezien hun depletie een toename in cytokineproductie

veroorzaakte zowel voor als na behandeling. In de meeste gevallen was celdeling veel minder sterk beïnvloed in vergelijking met de productie van verschillende cytokines.

We hebben ons daarna gericht op de rol van B cellen in het verloop van schistosoom-infectie. In **hoofdstuk 6** tonen we aan dat schistosomiasis leidt tot meer regulatoire B cellen (Breg), witte bloedcellen die net als regulatoire T cellen afweerreacties onderdrukken. We bewezen dat Gabonese schoolkinderen een verhoogd percentage $CD1d^{hi}$ Breg cellen in het bloed hebben en dat deze cellen meer van het onderdrukkende cytokine IL-10 produceerden. Een belangrijke bevinding was dat de frequentie van deze cellen was verlaagd na een schistosoom-behandeling tot een vergelijkbaar niveau als gevonden in niet-geïnfecteerde kinderen.

In **hoofdstuk 7** laten we zien dat naast veranderde Breg-frequenties, de frequentie van geheugen-B-cellen is toegenomen in geïnfecteerde schoolkinderen. Hoewel deze cellen meer geactiveerd lijken, zijn de celdeling en de productie van ontstekingsbevorderende cytokines in deze cellen verminderd. De behandeling van de kinderen voor hun wormen resulteerde grotendeels in functieherstel van deze cellen. De resultaten in dit hoofdstuk laten zien dat een infectie met *S. haematobium* leidt tot significante veranderingen in het B-cel-compartiment, zowel op fenotypisch als functioneel gebied.

Ten slotte worden in **hoofdstuk 8** de bevindingen van dit proefschrift samengevat. De interactie tussen aangeboren- en verworven afweerreacties en tolerantieprocessen worden besproken, waarbij de nadruk wordt gelegd op kenmerken van de tolerantieprocessen. Aangezien er is aangetoond dat tolerantieprocessen niet alleen belangrijk zijn voor chronische infectieziekten, maar ook voor chronische ontstekingsziekten, kan de kennis hier opgedaan van substantiële waarde zijn voor de gezondheid van diegenen die leiden aan infecties of aan ontstekingsziekten.

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