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

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General introduction.

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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

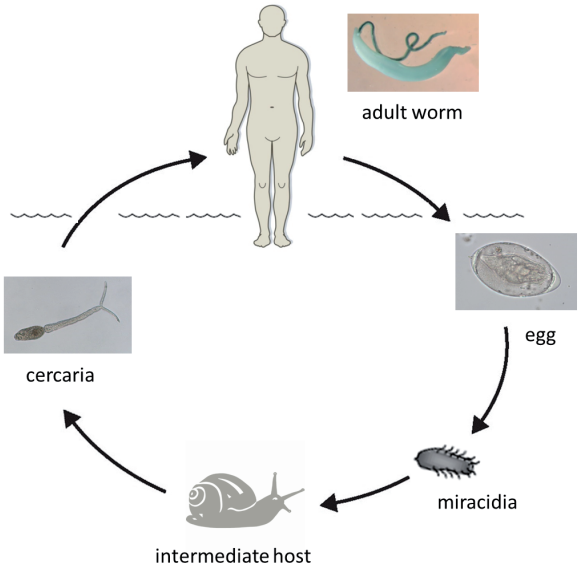

Figure 1: Life cycle of schistosomes
Snails are the intermediate host and humans the definitive hosts in the schistosome transmission cycle. Following penetration of the human skin the cercariae transform into schistosomula, which migrate in the blood stream through the lungs to the liver, where the 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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