The effect of parasitic co-infections on immune responses in Gabon: Particular emphasis on malaria and helminths

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„ Wisdom is like a baobab tree; no one individual can embrace it. “
African proverb

Contents

Chapter 1

General introduction

Helminth and malaria co-infection in humans: Where do we stand now?

I. Epidemiology of helminths and malaria co-infection

Parasitic helminths encompass a variety of different pathogens including filaria, schistosoma and intestinal helminths. They are widely distributed in tropical and sub-tropical areas where, as shown in figure 1, they share the same spatial distribution as malaria and usually co-infect the same human host (1,2).

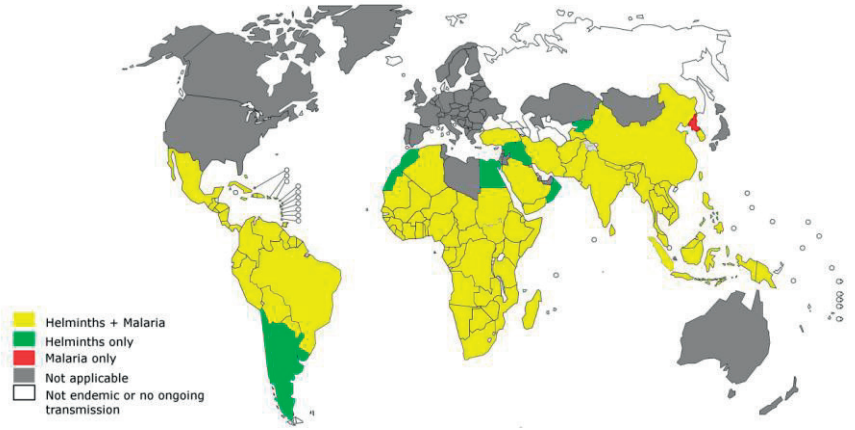

Figure 1: World map showing the geographical distribution of malaria and helminth infection or co-infection. Data on malaria are indicative of the percentage of population at risk in the world in 2013. These data were obtained from the WHO 2014 world malaria report. Data on helminths (lymphatic filariasis, onchocerciasis, schistosomiasis or soil-transmitted helminthiasis) were extracted from the following source (Lustigman, S. *et al*. A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis 6, e1582 (2012)).

Data are still lacking on the prevalence of malaria and helminth coinfection worldwide. However because more than 75% of malaria cases occur in sub-Saharan Africa (3), it is expected that this continent also bears the highest burden of helminths and malaria co-infection. Using a mathematical modelling approach it was recently estimated that approximately one quarter of African school-aged children were at risk of co-infection with malaria and hookworm (1). However because this study based its prediction on hookworm solely we can speculate

that a higher prevalence of co-infection would have been predicted if other helminth species were considered. Population-based studies conducted in different African countries have so far confirmed this (4–10) and have furthermore indicated an influence of helminths on malaria outcomes as depicted in Figure 2. For instance it was reported that helminth-infected individuals were more susceptible (5,6,11,12) or protected (7,8) against malaria and, had a higher (9,11) or a lower (10) carriage rate of malaria parasite depending on the species and the burden of helminth infection. Moreover in studies carried out in Thailand it was noted that malaria patients who were concurrently infected with helminths presented with less fever and were less likely to develop cerebral malaria or renal failure by comparison to their helminth-uninfected counterpart (13,14). However, there are contradictory findings in other settings (6,15). In addition, the effect of helminths on malaria induced anaemia, a leading cause of mortality in sub-Saharan Africa, has also been investigated. Although studies have suggested a protective effect of helminths on malaria-induced anaemia, a meta-analysis of all available published studies was not able to confirm these findings (16).

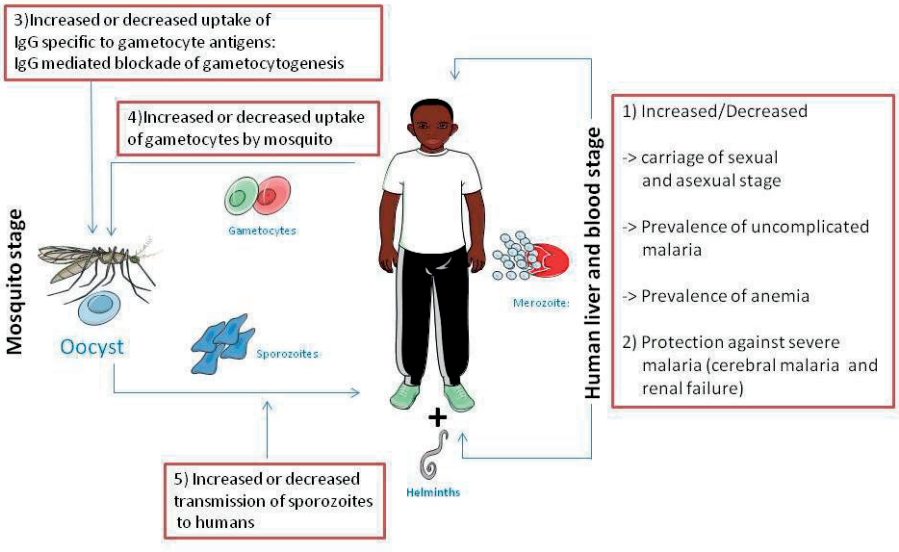

Figure 2: The possible influence of helminth infections on transmission and/or clinical outcomes of malaria parasites.

Many factors can influence the associations between helminths and malaria parasites or disease (Figure 3), among them helminth species involved infection pressure and burden, age of the study subjects and their nutritional status. *In utero* exposure to helminths antigens could also be added to this list (17,18). With respect to the latter, a birth cohort study conducted in Uganda showed that children who were followed from delivery till the age of five years had an increased risk of malaria infection and morbidity if their mother was infected with *M. perstans* and hookworm during pregnancy (19). Interestingly this result shows that not only current infection but also past exposure to helminth antigens might shape the host response to malaria parasite. Considerable efforts have been put into understanding the mechanisms whereby helminths can influence malaria infection. What is currently known is that helminths have the potential to manipulate the immune system of their host in order to ensure their survival, however, this immune modulation may have significant consequences on the host response to others pathogens such as *Plasmodium spp*.(20).

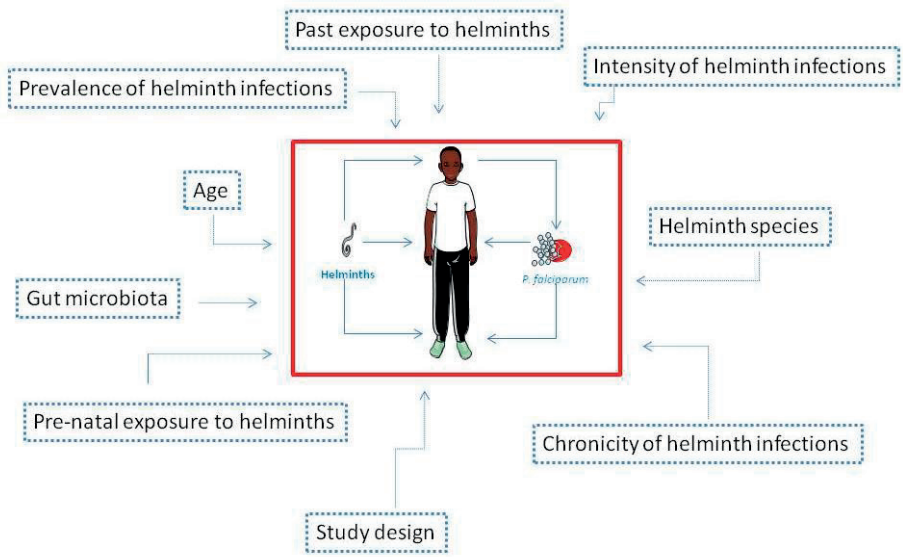

Figure 3: Infection with helminths or *P. falciparum* results in direct interaction between these parasites with the host immune system and metabolism. But co-infection can also result in direct or indirect interaction between these two parasites within the human host (depicted inside the red square). This interaction network might however be influenced by several factors shown here outside the red box. Because this thesis focuses on the effect of helminths on malaria we have emphasized the interference caused by helminths.

II. Immune response to *Plasmodium spp*. in the context of helminth co-infection

Learning from animal models

The acquisition of a protective immune response to *Plasmodium spp.* has proven to be a complex process. It depends on a well-adjusted balance between different effector mechanisms of the host immune system at different stages of the infection (21,22). During the acute phase of malaria, the host immune response is essentially cell mediated (23,24) and involves innate mechanisms that rely on the recognition and uptake of sporozoite by antigen presenting cells (25,26) and by the release of pro-inflammatory cytokines, primarily by γδ T cells as well as Natural killer (NK) and NK T cells (27). This is followed by essentially, but not exclusively, the involvement of CD8 T cells at the liver stage (28,29) along with a variety of CD4 helper T cells subsets when the parasite reaches the bloodstream (21,23). The adaptive immune response observed during the acute phase relies on a fine equilibrium between a panel of inflammatory and anti-inflammatory cytokines that aim at stopping the replication of the parasite on the one hand and preventing host tissue damage on the other (21,30–32). Understanding how helminths can influence anti-malaria immunity has been particularly of interest during the past decades. It is now well established that helminths have the potential to modulate the innate and adaptive immune response of their host (33,34). This immune modulation is characterized by an impairment of dendritic cell function (35), a Th2 skewed immune phenotype (36,37), and the induction of a potent regulatory network (38) leading to T cells hyporesponsiveness (39,40). In a mice model of *Plasmodium chabaudi* infection it was observed that the erythrocytic stage of *P. chabaudi* elicited a specific immune response characterized by the activation of IFN-γ producing T cells during the acute phase of the infection, followed by an increase of IL-4 producing T cells and of specific antibodies during the chronic phase. However in mice co-infected with *Heligosomoides polygirus* and *P. chabaudi* the presence of helminths resulted in a significantly lower production of IFN-γ along with an increase in the level of IL4, IL-10 and TGF-β in comparison to mice infected with *P. chabaudi* alone (41). As a consequence co-infected mice had a higher density of

parasitemia and higher mortality rate than those singly infected with *P. chabaudi* only (41). In contrast, a study by Karadjan and co-workers showed no effect of *Litosomoides sigmodontis* coinfection on the peak parasitemia of *P. chabaudi* whereas a decrease was observed for *Plasmodium yoelii* (42). Furthermore they did not report a difference in the level of IFN-γ or IL-10 between *P. yoelii* single infected mice and those co-infected (42). Such heterogeneity in outcomes have been attributed to differences in study methodology, model of malaria, helminth species and the genetic background of mice used (43). However in order to distil the current body of knowledge, and take into consideration these factors, a meta-analysis was conducted. The meta-analysis focused on all available articles describing murine models of helminth and malaria parasite co-infection where the major pro-inflammatory cytokine IFN-γ was assessed (44). The meta-analysis confirmed a helminth-induced decrease of IFN-γ in malaria infected mice despite the heterogeneity observed when single reports were considered. It showed that this effect correlated with an increase of parasite density in co-infected mice but it was independent of IL-4 (42). Unfortunately their analysis did not include anti-inflammatory cytokines or the regulatory cells which are known to be increased by helminths and can down modulate the pro-inflammatory cytokine responses in murine models of malaria (45). In general, the reported impairment of the host immune response induced by helminths reflects their ability to influence anti-malaria cell mediated immunity. This impairment results from a modulation exerted both on the innate and adaptive components of the immune system. It was for example shown that schistosome antigens can effectively modulate DCs (46). In a model of *H. polygyrus* and *P. chabaudi* co-infection, DCs from co-infected host were less able to induce *in vitro* proliferation or IFN-γ production by $CD4^+$ T cells in response to malaria antigen stimulation when compared to mice infected with *P. chabaudi* only (47). It should be noted that helminth infections can also result in intrinsic T cell hyporesponsiveness (48) as well as in induction of regulatory T (39,40) and regulatory B (49) cells able to dampen the function of effector T cells.

While the cellular immune response is important to reduce high patent parasitemia to low levels during the acute phase of malaria the final effective mechanism for clearance of blood stage parasites was suggested to be antibody dependent. It was indeed shown that B cell

knockout mice are unable to clear *P. chabaudi* despite their ability to reduce parasitemia during the acute infection (50). This situation led to a chronic relapsing parasitemia that was only controlled by a subsequent adoptive transfer of B cells. The role of *Plasmodium spp.* specific antibodies in anti-malaria immunity has been shown to range from blocking liver or erythrocyte invasion by parasites, preventing sequestration of infected erythrocytes in micro vasculature, mediating antibody dependent cellular immunity (ADCI) as well as preventing fertilisation of gametes (22). In the context of co-infection humoral response to *Plasmodium spp.* can be influenced by chronic helminth infections that are usually marked by Th2 skewing and regulatory responses along with an increased production of IgG4 and IgE (reviewed in (51,52)). This Th2 profile can interfere with the ADCI necessary to control *Plasmodium spp.* parasites, which rely on cytophilic antibodies (53,54). Experimental data are still scarce on how helminths influence B cells function and distribution in general, and more particularly in the context of malaria infection. Understanding this would be of particular importance given that new B cell subsets, such as regulatory B cells, implicated in immune tolerance (55), might help shed light on anti-malaria immunity.

Learning from population-based studies

Although studies in mice have been instrumental to understand how helminths can affect anti malaria specific immunity, they nevertheless represent a questionable model for helminth and malaria co-infections in humans (discussed in (56)). For this reason significant efforts have been devoted to determine the extent to which the findings in experimental animal models would translate into population-based studies. In helminth endemic countries where the force of the infection is relatively high, exposure to antigens can start in utero (18,57) and the first active helminth infection generally followed by repeated re-infection, happen early in life (58,59) leading to chronicity at a relatively young age. In the case of *S. haematobium*, chronic infection is marked by the release of eggs by the female worms into the bladder that cause local damage resulting in haematuria. From the perspective of the host immune response, chronic *S. haematobium* infection is characterized by a Th2 skewed immune phenotype (60) that leads to B-cells class switching towards IgG4 and IgE. The balance between these

two antibodies is thought to be important in anti-schistosoma immunity, contributing to resistance or susceptibility to re-infection. When considered in detail, studies have indicated that schistosome antigen specific IgM, IgE, IgA and IgG1 are rather protective against re-infection whereas IgG2 and IgG4 are mainly associated with susceptibility (61–64). Although the role of humoral immune response in anti-schistosoma immunity is well understood it is not very clear why it takes years for naturally exposed individuals to develop a protective antibody response. While this might result from a general state of hyporesponsiveness mediated by regulatory cells (65), a helminth induced impairment of B cell function cannot be formally ignored and needs to be assessed given the existence of reports indicating an increased proportion of exhausted B cells in individuals with chronic viral infections (66,67). Addressing this question might also help to extend our knowledge on how helminths interfere with anti-malaria immunity.

Epidemiological studies conducted in endemic areas have investigated the influence of helminths on either the cellular or the humoral immune response of individuals co-infected with *P. falciparum*. Unfortunately these studies have so far generated contradictory results that are difficult to reconcile. For example infection with *S. haematobium* in malaria co-infected children in Senegal was associated with an increase of IgG1 and IgG3 cytophylic antibodies specific to $MSP1_{19}$ *P. falciparum* antigen (68) whereas in Mali it did not influence the level of total IgG to AMA1 or MSP1 (69) and in Zimbabwe it was negatively correlated with IgG1, IgG4 and IgM antibodies against schizont extracts, MSP3 and GLURP (70). More studies are still needed to get a comprehensive picture of the influence of helminths on anti-malaria humoral immunity. It will also be important to determine the effect of helminths on *P. falciparum* sexual stage antibody responses. Indeed the few studies that are currently available have only focused on antibody response to *P. falciparum* asexual stage antigens whereas some data indicate that carriage of *P. falciparum* gametocytes can be increased in helminth infected subjects (71,72) possibly as a consequence of an impairment of the host immunity to gametocyte antigens. An effect of helminths has also been reported on the cellular immune responses of malaria infected subjects. However its direction is still a matter of debate. In the case of the Th1 mediated anti-malaria immunity, some studies reported an increase (73,74) or a decrease (74,75) in the level of

Th1/pro inflammatory cytokines such as IFN-γ and TNF in helminths and malaria co-infected subjects. However, there are also studies that show no association between helminths and immune responses to *P. falciparum* infection(76–78). Similar discrepancies have similarly been observed in studies where other arms of anti- malarial cellular immunity such as the Th2, Th17 or Regulatory T cell responses were assessed. It is possible that the observed differences in study outcomes reflect underlying heterogeneities in the study sites and methodologies: 1) different epidemiological features of malaria and helminth infections; 2) age and history of exposure to infection; 3) other helminth infections not considered. As indicated in table 1 all the current available immuno-epidemiological studies were also characterized by the diversity of immunological assays used to assess the influence of helminths on cellular immune responses during *P. falciparum* infections. For example, many different methods were used to measure the cytokines involved, in plasma, in serum, in supernatants or intra-cellularly following stimulation of peripheral blood mononuclear cells or whole blood by different stimuli. These differences might explain, to some extent, the observed discrepancies in the direction of the effect of helminths on cellular immunity upon *P. falciparum* infection. It is also important to underline that in most cases studies on this topic have only given an incomplete picture of the interaction between helminths and cellular immunity during malaria by focusing on only few cytokines or few parameters to characterise the Th1, Th2, Th17 or regulatory T cells immune responses. Adding to this is our limited knowledge of innate immunity in the context of co-infection. Majority of studies have indeed focused on malaria specific adaptive immunity and none have so far assessed both the innate and adaptive immune responses in the same cohort.

I. Scope and aims of this thesis

The main objective of this thesis is to improve our understanding of helminth and *Plasmodium spp.* co-infection within their human host. We aimed to assess how helminths manipulate the immune system of their human host and how such a manipulation could affect immune response in subjects infected with malaria parasites. Our study population was selected in an area where the burden of helminths (particularly *S. haematobium*, filaria, intestinal helminths) and malaria

Table 1: Summary table of epidemiological studies that assessed the effect of helminth infections on the immune response of individuals infected with *P. falciparum*

N°	Authors (year of publication)	Type of immunological assay performed	Type of stimuli used if cells were stimulated	Type of read out
1	Noone et al. (2013)	Plasma cytokine	NA	Plasma cytokines
2	Courtin et al.	Plasma cytokine	NA	Plasma cytokines
3	Diallo et al. (2010)	Whole blood culture	MSP1 antigens and *P. falciparum* schizont lysate	Cytokines measured in the cell culture supernatant
4	Dolo et al. (2012)	Plasma cytokine	NA	Plasma cytokine
5	Hartgers et al. (2009)	Whole blood culture	*P. falciparum* infected red bllod cells (iRBCs)	Cytokines measured in the cell culture supernatant
6	Lyke et al. (2006)	Serum cytokine	NA	Serum cytokine
7	Metenou et al. (2009)	Whole blood culture	iRBCs	Cytokines measured in cell culture supernatant
8	Metenou et al. (2011)	Whole blood culture	iRBCs	Intracellular cytokines
9	Muok et al. (2009)	Whole blood collected and stained withouth stimulation		T cells characterized by surface markers
10	Nmorsi (2009)	Plasma cytokines	NA	Plasma cytokines
11	Wilson et al. (2009)	Plasma cytokines	NA	Plasma cytokines
12	Lyke et al. (2012)	Surface staining of PBMC	NA	T cells characterized by surface markers
13	Metenou et al. (2012)	Whole blood culture Gene expression cDNA synthesis RT-PCR	iRBCs	RNA mDCs, pDCs
14	Panda et al. (2013)	Plasma cytokine Whole blood assay	NA	Plasma cytokine Regulatory T cells characterized by surface markers
15	Wammes et al. (2010)	Cell isolation, depletion and phenotyping Proliferation assay	iRBCs	T cells characterization T cells proliferation Cytokines in cell culture supernatatant
16	Wilson et al. (2008)	Whole blood culture	SEA, SWA and PHA	Cytokines in cell culture supernatatant
17	Hartgers et al. (2008)	Whole blood culture	iRBCs	Cytokines measured in the cell culture supernatant
18	Lyke et al. (2012)	PBMC culture Memory B cells assays ELISA Flow cytometry staining ELISPOT	SEA, SWAP, AMA1, MSP1	Antibodies, Memory B cells response to malaria or schistosoma antigens
19	Diallo et al. (2004)	Plasma cytokines	NA	Plasma cytokines

are remarkably high. Characterizing immune responses of subjects living within this context is of special interest. Indeed whereas experimental studies inform us on how the immune system works in optimal conditions, field studies involving naturally exposed individuals might give a valuable insight into the immunological profiles encountered in endemic regions. We set out to assess the different arms of the immune system. On the one hand we assess the humoral response to *P. falciparum* sexual and asexual stages antigens and the effect of *S. haematobium* on B cell subsets and function. On the other, we evaluate the cellular immune reactivity by analysing the host innate and adaptive immune response in *P. falciparum* infected subjects in the context of concurrent chronic helminth infections.

II. Outline of this thesis

This thesis is divided into height chapters:

The **first chapter** serves as Introduction.

The **second chapter** describes the study population, the study area, as well as the study procedures and different immunological assays that were used.

In the **third chapter** we investigate the effect of *S. haematobium* infection on the different B cells subsets and their response to B cell receptor engagement and TLR ligands.

In the **fourth chapter** we expand our studies to assess the effect of helminths on the antibody response specific to *P. falciparum* sexual and asexual stage antigens.

In the **fifth chapter** we assess the *in utero* effect of filarial infection on the Th1, Th17 and regulatory T cell responses in newborns.

In the **sixth chapter** the innate and adaptive immune responses of *S. haematobium* and *P. falciparum* co-infected school age children were compared to children with *P. falciparum* infection only.

The **seventh chapter** consist of a meta-analysis that extends our assessment of the effect of helminths on the cellular immune responses of subjects infected with *Plasmodium spp.* to all available results of such studies in the literature.

Finally in the **eighth chapter** we discuss the main findings of this thesis.

REFERENCES

1.Brooker S, Clements AC, Hotez PJ, Hay SI, Tatem AJ, Bundy DA, et al. The co-distribution of Plasmodium falciparum and hookworm among African schoolchildren. Malar J. 2006 Nov 3;5(1):99.

2.Brooker SJ, Pullan RL, Gitonga CW, Ashton RA, Kolaczinski JH, Kabatereine NB, et al. Plasmodium-helminth coinfection and its sources of heterogeneity across East Africa. J Infect Dis. 2012 Mar 1;205(5):841–52.

3.WHO | World Malaria Report 2014 [Internet]. [cited 2015 Nov 17]. Available from: http://www.who.int/malaria/publications/world_malaria_report_2014/en/

4.Mulu A, Legesse M, Erko B, Belyhun Y, Nugussie D, Shimelis T, et al. Epidemiological and clinical correlates of malaria-helminth co-infections in Southern Ethiopia. Malar J. 2013;12:227.

5.Kepha S, Nuwaha F, Nikolay B, Gichuki P, Edwards T, Allen E, et al. Epidemiology of coinfection with soil transmitted helminths and Plasmodium falciparum among school children in Bumula District in western Kenya. Parasit Vectors. 2015;8(1):314.

6.Degarege A, Legesse M, Medhin G, Animut A, Erko B. Malaria and related outcomes in patients with intestinal helminths: a cross-sectional study. BMC Infect Dis. 2012;12:291.

7.Lyke KE, Dicko A, Dabo A, Sangare L, Kone A, Coulibaly D, et al. Association of Schistosoma haematobium infection with protection

against acute Plasmodium falciparum malaria in Malian children. Am J Trop Med Hyg. 2005 Dec;73(6):1124–30.

8.Lemaitre M, Watier L, Briand V, Garcia A, Le Hesran JY, Cot M. Coinfection with Plasmodium falciparum and Schistosoma haematobium: additional evidence of the protective effect of Schistosomiasis on malaria in Senegalese children. Am J Trop Med Hyg. 2014 Feb;90(2):329–34.

9.Nkuo-Akenji TK, Chi PC, Cho JF, Ndamukong KKJ, Sumbele I. Malaria and helminth co-infection in children living in a malaria endemic setting of mount Cameroon and predictors of anemia. J Parasitol. 2006 Dec;92(6):1191–5.

10.Kinung'hi SM, Magnussen P, Kaatano GM, Kishamawe C, Vennervald BJ. Malaria and helminth co-infections in school and preschool children: a cross-sectional study in Magu district, north-western Tanzania. PloS One. 2014;9(1):e86510.

11.Mulu A, Legesse M, Erko B, Belyhun Y, Nugussie D, Shimelis T, et al. Epidemiological and clinical correlates of malaria-helminth co-infections in Southern Ethiopia. Malar J. 2013;12:227.

12.Nacher M, Singhasivanon P, Yimsamran S, Manibunyong W, Thanyavanich N, Wuthisen R, et al. Intestinal helminth infections are associated with increased incidence of Plasmodium falciparum malaria in Thailand. J Parasitol. 2002 Feb;88(1):55–8.

13.Nacher M, Singhasivanon P, Silachamroon U, Treeprasertsuk S, Vannaphan S, Traore B, et al. Helminth infections are associated with protection from malaria-related acute renal failure and jaundice in Thailand. Am J Trop Med Hyg. 2001 Dec;65(6):834–6.

14.Nacher M, Singhasivanon P, Traore B, Dejvorakul S, Phumratanaprapin W, Looareesuwan S, et al. Short report: Hookworm infection is associated with decreased body temperature during mild Plasmodium falciparum malaria. Am J Trop Med Hyg. 2001 Aug;65(2):136–7.

15.Panda M, Sahoo PK, Mohapatra AD, Dutta S kanti, Thatoi PK, Tripathy R, et al. Decreased prevalence of sepsis but not mild or severe P. falciparum malaria is associated with pre-existing filarial infection. Parasit Vectors. 2013;6:203.

16.Naing C, Whittaker MA, Nyunt-Wai V, Reid SA, Wong SF, Mak JW, et al. Malaria and soil-transmitted intestinal helminth co-infection and its effect on anemia: a meta-analysis. Trans R Soc Trop Med Hyg. 2013 Nov;107(11):672–83.

17.Bal MS, Mandal NN, DAS MK, Kar SK, Sarangi SS, Beuria MK. Transplacental transfer of filarial antigens from Wuchereria bancrofti-infected mothers to their offspring. Parasitology. 2010 Apr;137(4):669–73.

18.Pires ML, Ferreira RS, Araújo S, Albuquerque R, Remigio Neto J, Da Silva AS, et al. [Transplacental passage of Wuchereria bancrofti microfilariae in newborns of microfilaremic mothers]. Rev Inst Med Trop São Paulo. 1991 Apr;33(2):91–5.

19.Ndibazza J, Webb EL, Lule S, Mpairwe H, Akello M, Oduru G, et al. Associations between maternal helminth and malaria infections in pregnancy and clinical malaria in the offspring: a birth cohort in entebbe, Uganda. J Infect Dis. 2013 Dec 15;208(12):2007–16.

20.Metenou S, Babu S, Nutman TB. Impact of filarial infections on coincident intracellular pathogens: Mycobacterium tuberculosis and Plasmodium falciparum. Curr Opin HIV AIDS. 2012 May;7(3):231–8.

21.Perez-Mazliah D, Langhorne J. CD4 T-cell subsets in malaria: TH1/TH2 revisited. Front Immunol. 2014;5:671.

22.Stevenson MM, Riley EM. Innate immunity to malaria. Nat Rev Immunol. 2004 Mar;4(3):169–80.

23.Taylor-Robinson AW, Phillips RS. Functional characterization of protective CD4+ T-cell clones reactive to the murine malaria parasite Plasmodium chabaudi. Immunology. 1992 Sep;77(1):99–105.

24.Taylor-Robinson AW, Phillips RS. Reconstitution of B-cell-depleted mice with B cells restores Th2-type immune responses during Plasmodium chabaudi chabaudi infection. Infect Immun. 1996 Jan;64(1):366–70.

25.Perry JA, Rush A, Wilson RJ, Olver CS, Avery AC. Dendritic cells from malaria-infected mice are fully functional APC. J Immunol Baltim Md 1950. 2004 Jan 1;172(1):475–82.

26.Ing R, Segura M, Thawani N, Tam M, Stevenson MM. Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. J Immunol Baltim Md 1950. 2006 Jan 1;176(1):441–50.

27.D'Ombrain MC, Hansen DS, Simpson KM, Schofield L. gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to Plasmodium falciparum malaria. Eur J Immunol. 2007 Jul;37(7):1864–73.

28.Nganou-Makamdop K, van Gemert G-J, Arens T, Hermsen CC, Sauerwein RW. Long Term Protection after Immunization with P. berghei Sporozoites Correlates with Sustained IFNγ Responses of Hepatic CD8+ Memory T Cells. PLoS ONE [Internet]. 2012 May 1 [cited 2015 Nov 18];7(5). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3341355/

29.Schmidt NW, Podyminogin RL, Butler NS, Badovinac VP, Tucker BJ, Bahjat KS, et al. Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. Proc Natl Acad Sci U S A. 2008 Sep 16;105(37):14017–22.

30.Hanum P S, Hayano M, Kojima S. Cytokine and chemokine responses in a cerebral malaria-susceptible or -resistant strain of mice to Plasmodium berghei ANKA infection: early chemokine expression in the brain. Int Immunol. 2003 May;15(5):633–40.

31.Nie CQ, Bernard NJ, Schofield L, Hansen DS. CD4+ CD25+ regulatory T cells suppress CD4+ T-cell function and inhibit the development of Plasmodium berghei-specific TH1 responses involved

in cerebral malaria pathogenesis. Infect Immun. 2007 May;75(5):2275–82.

32.Schmidt KE, Schumak B, Specht S, Dubben B, Limmer A, Hoerauf A. Induction of pro-inflammatory mediators in Plasmodium berghei infected BALB/c mice breaks blood-brain-barrier and leads to cerebral malaria in an IL-12 dependent manner. Microbes Infect Inst Pasteur. 2011 Sep;13(10):828–36.

33.van Riet E, Hartgers FC, Yazdanbakhsh M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. Immunobiology. 2007;212(6):475–90.

34.Everts B, Smits HH, Hokke CH, Yazdanbakhsh M. Helminths and dendritic cells: sensing and regulating via pattern recognition receptors, Th2 and Treg responses. Eur J Immunol. 2010 Jun;40(6):1525–37.

35.Everts B, Adegnika AA, Kruize YCM, Smits HH, Kremsner PG, Yazdanbakhsh M. Functional impairment of human myeloid dendritic cells during Schistosoma haematobium infection. PLoS Negl Trop Dis. 2010;4(4):e667.

36.Everts B, Perona-Wright G, Smits HH, Hokke CH, van der Ham AJ, Fitzsimmons CM, et al. Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. J Exp Med. 2009 Aug 3;206(8):1673–80.

37.Vella AT, Pearce EJ. CD4+ Th2 response induced by Schistosoma mansoni eggs develops rapidly, through an early, transient, Th0-like stage. J Immunol Baltim Md 1950. 1992 Apr 1;148(7):2283–90.

38.Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, et al. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis. 2012;6(5):e1655.

39.Wammes LJ, Hamid F, Wiria AE, de Gier B, Sartono E, Maizels RM, et al. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. Eur J Immunol. 2010 Feb;40(2):437–42.

40.Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, Maizels RM. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. J Immunol Baltim Md 1950. 2005 Apr 15;174(8):4924–33.

41.Su Z, Segura M, Morgan K, Loredo-Osti JC, Stevenson MM. Impairment of protective immunity to blood-stage malaria by concurrent nematode infection. Infect Immun. 2005 Jun;73(6):3531–9.

42.Karadjian G, Berrebi D, Dogna N, Vallarino-Lhermitte N, Bain O, Landau I, et al. Co-infection restrains Litomosoides sigmodontis filarial load and plasmodial P. yoelii but not P. chabaudi parasitaemia in mice. Parasite Paris Fr. 2014;21:16.

43. Knowles SCL. The effect of helminth co-infection on malaria in mice: a meta-analysis. Int J Parasitol. 2011 Aug 15;41(10):1041–51.

44. Graham AL. Ecological rules governing helminth-microparasite coinfection. Proc Natl Acad Sci U S A. 2008 Jan 15;105(2):566–70.

45. Wang M, Cao Y, Luo E, Zhang Y, Guo Y. Pre-existing Schistosoma japonicum infection alters the immune response to Plasmodium berghei infection in C57BL/6 mice. Malar J. 2013;12:322.

46. Aksoy E, Zouain CS, Vanhoutte F, Fontaine J, Pavelka N, Thieblemont N, et al. Double-stranded RNAs from the helminth parasite Schistosoma activate TLR3 in dendritic cells. J Biol Chem. 2005 Jan 7;280(1):277–83.

47. Su Z, Segura M, Morgan K, Loredo-Osti JC, Stevenson MM. Impairment of protective immunity to blood-stage malaria by concurrent nematode infection. Infect Immun. 2005 Jun;73(6):3531–9.

48.Taylor JJ, Krawczyk CM, Mohrs M, Pearce EJ. Th2 cell hyporesponsiveness during chronic murine schistosomiasis is cell intrinsic and linked to GRAIL expression. J Clin Invest. 2009 Apr;119(4):1019–28.

49. van der Vlugt LEPM, Zinsou JF, Ozir-Fazalalikhan A, Kremsner PG, Yazdanbakhsh M, Adegnika AA, et al. Interleukin 10 (IL-10)-

producing CD1dhi regulatory B cells from Schistosoma haematobium-infected individuals induce IL-10-positive T cells and suppress effector T-cell cytokines. J Infect Dis. 2014 Oct 15;210(8):1207–16.

50. Langhorne J, Cross C, Seixas E, Li C, von der Weid T. A role for B cells in the development of T cell helper function in a malaria infection in mice. Proc Natl Acad Sci U S A. 1998 Feb 17;95(4):1730–4.

51. Bouchery T, Kyle R, Ronchese F, Le Gros G. The Differentiation of CD4+ T-Helper Cell Subsets in the Context of Helminth Parasite Infection. Front Immunol [Internet]. 2014 Oct 15 [cited 2015 Nov 18];5. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4197778/

52.van Riet E, Hartgers FC, Yazdanbakhsh M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. Immunobiology. 2007;212(6):475–90.

53.Green TJ, Kreier JP. Demonstration of the role of cytophilic antibody in resistance to malaria parasites (Plasmodium berghei) in rats. Infect Immun. 1978 Jan;19(1):138–45.

54.Bouharoun-Tayoun H, Druilhe P. Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. Infect Immun. 1992 Apr;60(4):1473–81.

55.Lund FE, Garvy BA, Randall TD, Harris DP. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. Curr Dir Autoimmun. 2005;8:25–54.

56.Salazar-Castañon VH, Legorreta-Herrera M, Rodriguez-Sosa M. Helminth Parasites Alter Protection against Plasmodium Infection. BioMed Res Int [Internet]. 2014 [cited 2015 Jul 20];2014. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4170705/

57.Anosike JC, Onwuliri CO, Abanobi OC. A case of probable transplacental transmission of Wuchereria bancrofti microfilariae. Appl Parasitol. 1994 Nov;35(4):294–6.

58.Davis SM, Worrell CM, Wiegand RE, Odero KO, Suchdev PS, Ruth LJ, et al. Soil-Transmitted Helminths in Pre-School-Aged and School-Aged Children in an Urban Slum: A Cross-Sectional Study of Prevalence, Distribution, and Associated Exposures. Am J Trop Med Hyg. 2014 Nov 5;91(5):1002–10.

59.Belyhun Y, Medhin G, Amberbir A, Erko B, Hanlon C, Alem A, et al. Prevalence and risk factors for soil-transmitted helminth infection in mothers and their infants in Butajira, Ethiopia: a population based study. BMC Public Health. 2010;10:21.

60.Pearce EJ, M Kane C, Sun J, J Taylor J, McKee AS, Cervi L. Th2 response polarization during infection with the helminth parasite Schistosoma mansoni. Immunol Rev. 2004 Oct;201:117–26.

61.Caldas IR, Correa-Oliveira R, Colosimo E, Carvalho OS, Massara CL, Colley DG, et al. Susceptibility and resistance to Schistosoma mansoni reinfection: parallel cellular and isotypic immunologic assessment. Am J Trop Med Hyg. 2000 Jan;62(1):57–64.

62.Demeure CE, Rihet P, Abel L, Ouattara M, Bourgois A, Dessein AJ. Resistance to Schistosoma mansoni in humans: influence of the IgE/IgG4 balance and IgG2 in immunity to reinfection after chemotherapy. J Infect Dis. 1993 Oct;168(4):1000–8.

63.Hagan P, Blumenthal UJ, Dunn D, Simpson AJ, Wilkins HA. Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium. Nature. 1991 Jan 17;349(6306):243–5.

64. Vereecken K, Naus CWA, Polman K, Scott JT, Diop M, Gryseels B, et al. Associations between specific antibody responses and resistance to reinfection in a Senegalese population recently exposed to Schistosoma mansoni. Trop Med Int Health TM IH. 2007 Mar;12(3):431–44.

65.Yazdanbakhsh M, Sacks DL. Why does immunity to parasites take so long to develop? Nat Rev Immunol. 2010 Feb;10(2):80–1.

66.Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. -

PubMed - NCBI [Internet]. [cited 2015 Dec 22]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18625747/

67.Clonal expansion of immunoglobulin M+CD27+ B cells in HCV-associated mixed cryoglobulinemia. - PubMed - NCBI [Internet]. [cited 2015 Dec 22]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17942751/

68.Diallo TO, Remoue F, Gaayeb L, Schacht A-M, Charrier N, De Clerck D, et al. Schistosomiasis coinfection in children influences acquired immune response against Plasmodium falciparum malaria antigens. PloS One. 2010;5(9):e12764.

69.Lyke KE, Wang A, Dabo A, Arama C, Daou M, Diarra I, et al. Antigen-specific B memory cell responses to Plasmodium falciparum malaria antigens and Schistosoma haematobium antigens in co-infected Malian children. PloS One. 2012;7(6):e37868.

70.Mutapi F, Roussilhon C, Mduluza T, Druilhe P. Anti-malaria humoral responses in children exposed to Plasmodium falciparum and Schistosoma haematobium. Mem Inst Oswaldo Cruz. 2007 Jun;102(3):405–9.

71.Nacher M, Singhasivanon P, Silachamroon U, Treeprasertsu S, Krudsood S, Gay F, et al. Association of helminth infections with increased gametocyte carriage during mild falciparum malaria in Thailand. Am J Trop Med Hyg. 2001 Nov;65(5):644–7.

72.Sangweme DT, Midzi N, Zinyowera-Mutapuri S, Mduluza T, Diener-West M, Kumar N. Impact of schistosome infection on Plasmodium falciparum Malariometric indices and immune correlates in school age children in Burma Valley, Zimbabwe. PLoS Negl Trop Dis. 2010;4(11):e882.

73.Noone C, Parkinson M, Dowling DJ, Aldridge A, Kirwan P, Molloy SF, et al. Plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with Plasmodium falciparum. Malar J. 2013;12:5.

74.Nmorsi O, Isaac C, Ukwandu N, Ekundayo A, Ekozien M. Schistosoma haematobium and Plasmodium falciparum co-infection with protection against Plasmodium falciparum malaria in Nigerian children. Asian Pacific journal of tropical medicine. :16–20.

75.Metenou S, Dembele B, Konate S, Dolo H, Coulibaly YI, Diallo AA, et al. Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol Baltim Md 1950. 2011 Apr 15;186(8):4725–33.

76.Diallo TO, Remoue F, Schacht AM, Charrier N, Dompnier J-P, Pillet S, et al. Schistosomiasis co-infection in humans influences inflammatory markers in uncomplicated Plasmodium falciparum malaria. Parasite Immunol. 2004 Sep;26(8-9):365–9.

77. Dolo H, Coulibaly YI, Dembele B, Konate S, Coulibaly SY, Doumbia SS, et al. Filariasis attenuates anemia and proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults. PLoS Negl Trop Dis. 2012;6(11):e1890.

78.Wilson S, Jones FM, Mwatha JK, Kimani G, Booth M, Kariuki HC, et al. Hepatosplenomegaly associated with chronic malaria exposure: evidence for a pro-inflammatory mechanism exacerbated by schistosomiasis. Parasite Immunol. 2009 Feb;31(2):64–71.

Chapter 2

Assessment of the effect of *Schistosoma haematobium* co infection on malaria parasites and immune responses in rural populations in Gabon: study protocol

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Abstract

Background

Malaria and helminth co infection are common in tropical and subtropical areas where they affect the life of millions of people. While both helminth and malaria parasites have immunomodulatory activities, little is known about the consequence of co-infections on malaria antigen specific immune responses.

Method/Design

This study will be conducted in two rural areas of the Moyen Ogooué province in Gabon, endemic for both *Plasmodium falciparum* and *Schistosoma haematobium* infections. Participants, 5 to 50 years old, will be enrolled and grouped according to their infection status. *S. haematobium* and malaria parasites will be detected, demographic and clinical data will be recorded and blood will be collected for hematological as well as for immunological assays. The level of antibody specific to *Plasmodium falciparum* blood stage and gametocyte antigens will be measured using ELISA. PBMC will be isolated for phenotyping of different T cell subsets *ex vivo* by flow cytometry and for culture and cytokine response assessment.

Discussion

We will provide a comprehensive picture of the interaction between schistosomes and malaria parasites which co-localize in peripheral blood. We will test the hypothesis that schistosome infection has an impact on specific humoral as well as on cellular immune responses to malaria antigens.

Keywords

Malaria, Helminths, Co-infection, Cellular immune response, Study protocols, Epidemiology, Gabon, Africa

Background

Malaria and helminth infections are two of the major causes of mortality and morbidity in developing countries (Brooker et al. 2007; Hotez and Kamath 2009; Mwangi et al. 2006). Both infections are highly endemic in tropical and sub tropical areas (Adegnika and Kremsner 2012; Akue et al. 2011; Brooker et al. 2007). In the tropics, Sub Saharan Africa (SSA) bears the heaviest burden of *Plasmodium spss.* infection occurring mainly in children under five years. Moreover it is reported that almost 90% of all schistosomiasis cases worldwide are confined into this part of the world (Hotez and Kamath 2009; Simoonga et al. 2009). In developing countries, infection with multiple species of parasites is often the norm (Griffiths et al. 2011; Raso et al. 2004).

Parasitic coinfection is a relatively new research area. Although some data have been generated, much is unknown and contradictions persist on the impact of helminth infections on malarial disease or parasitemia during co-infection (Adegnika and Kremsner 2012; Brooker et al. 2007; Hartgers and Yazdanbakhsh 2006; Nacher 2011). At the clinical level, interaction between plasmodium and helminth species has been discussed; while some studies have highlighted the protective effect of helminth infection on severe malaria and its association with a decreased incidence of malaria attacks or malaria parasite density (Boel et al. 2010; Lemaitre et al. 2014; Nacher et al. 2000), other studies have given a completely opposite picture (Le Hesran et al. 2004; Sangweme et al. 2010). It seems that the outcome of the interaction between helminth and malaria is helminth species specific with, for example, Ascaris infection more likely to be protective against severe forms of malaria and infection with hookworm associated with an increase of malaria incidence (Adegnika and Kremsner 2012; Nacher 2011). However despite these opinions more data are needed to get a clear picture of the situation.

Immunity and pathology to malaria is thought to be dependent on a balance between different arms of the immune system. Indeed, whereas at the early stages of infection, the presence of *Plasmodium spp.* in the blood stream is associated with the production of proinflammatory

cytokines, activated cytotoxic T cells and γδ T cells, the effective clearance of the parasite is thought to be mediated by cytophilic antibodies of the IgG1 and IgG3 isotypes (Bouharoun-Tayoun and Druilhe 1992; Hartgers and Yazdanbakhsh 2006; Langhorne et al. 1998; Leoratti et al. 2008). However, the hallmark of immune responses during chronic helminth infections is the strong polarization toward Th2 and the downstream production of IgE and IgG4 antibodies. This Th2 skewed response is followed by the activation of an immunoregulatory network which can lead to cellular hyporesponsiveness with limited cells proliferation and cytokine production (Hartgers and Yazdanbakhsh 2006; Maizels and Yazdanbakhsh 2003; Nacher 2011). Down regulation of the immune response has been shown to be important for the survival of the parasite and for the restriction of deleterious immune response that lead to tissue pathology in the host (Belkaid 2007; Maizels and Smith 2011).

It is hypothesized that chronic helminth infections, with their marked immunomodulatory properties are able to modify immune responses to antigens derived from other pathogens (Hartgers and Yazdanbakhsh 2006; Maizels and Yazdanbakhsh 2003). This has been studied for helminth and malaria coinfection but again with conflicting results. For example, studies reported that schistosome infections decrease (Courtin et al. 2011) or favor (Remoue et al. 2003) the production of cytophilic antibodies protective against *Plasmodium falciparum* malaria, while another study in Zimbabwe, reported no association between *Schistosoma* infection and humoral response to malaria parasites (Sangweme et al. 2010). Inconclusive results were also reported when assessing cytokine productions in malaria co-infected subjects (Sangweme et al. 2010). In two different studies undertaken in Ghana and Mali, IL-10 responses to malaria antigen were found to be higher in helminth and malaria co-infected subjects (Hartgers et al. 2009; Lyke et al. 2012) whereas in a study from Senegal the level of INFγ was higher in co-infected subjects and the increase of IL-10 was only observed in adults but not in children when considering schistosoma and malaria co-infection (Diallo et al. 2004). Helminth infections have also been thought to increase malaria transmission intensity (MTI), as demonstrated in two studies showing an increase of gametocyte carriage in helminth infected subjects in comparison to non-infected ones (Nacher et al. 2001; Sangweme et al. 2010). Interestingly, using a

murine model of malaria and helminth coinfection, Noland *et al.* observed that anopheles mosquitoes exposed to co-infected mice had a higher rate of infectivity than those exposed to malaria only infected mice (Noland et al. 2007). The mechanism behind the possible impact of helminths on malaria transmission is still unclear,

Taking into account the reports in literature, we set out to conduct a study in an area where *S. haematobium* and *P. falciparum* infections are highly endemic (Adegnika et al. 2010; Wildling et al. 1995). The study will test the hypothesis that active helminth infections alter the humoral and cellular immune response to *Plasmodium falciparum* antigens. A global picture of both the antibody signature and the cytokine profiles will be obtained as well as the activation status of B Cells, T cells, monocytes and dendritic cells. Furthermore the role of regulatory T cells will be assessed by functional analysis, using Treg depletion strategies and comparison of immune responses to depleted and Treg containing cell fractions.

Methods/design

1. Study site

The study has taken place in two distinct areas, the Bindo village and the Pk15 area, located in the Moyen-Ogooué province of Gabon, central Africa. Several lakes are present in this district and the temperature has an average of 27°C. The capital of the Moyen-Ogooué province is Lambaréné, a semi urban town of about 35.000 inhabitants surrounded by villages. Bindo and the Pk15 are respectively 60 and 15 kilometers from Lambaréné (Figure 1) and they similarly present all the characteristics of a rural area.

Bindo village is a relatively remote place with around 1000 inhabitants. It is located in the center of a palm tree plantation. The income is generated mainly from work at the palm plantation owned by a private company. Few inhabitants get their income from fishing activities and almost all of the population grows their own food. The water is supplied directly from the Ogooué River to two public taps in the

village through a pipeline. The village has one nursery, one primary school and one small shop which sell manufactured products.

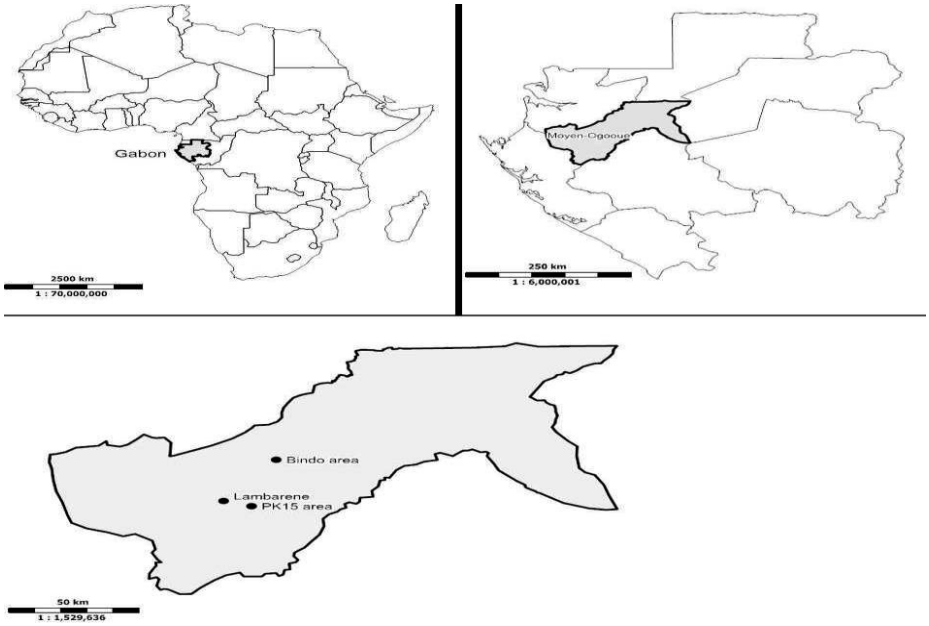

Figure 1: **Map representing the localization of the study area (lower panel).** The upper panel shows the emplacement of Gabon in Africa (in the left) and the localization of the Moyen Ogooué province in Gabon (in the right).

In contrast to Bindo, the Pk15 area is closer to Lambaréné; it is stretched along a national single lane highway over a 30 km distance. The population is very mobile and due to the proximity to Lambaréné the source of the income is diverse although most of the inhabitants work in an industrial palm oil plantation and engage in agriculture and fishing activities for their subsistence. Four primary schools and two nurseries are present in the Pk15 area. The area has neither electricity nor tap water and drinkable water is taken from streams neighboring the houses.

The streams both in the Pk15 area and Bindo represent the main source of drinking water. They are the sites for laundry and bathing but also represent important points for contact with *S. haematobium* which has been observed to be highly prevalent in these areas. Furthermore, numerous studies have shown that malaria, intestinal helminth and

filarial infections are prevalent in these areas (Adegnika et al. 2010; Wildling et al. 1995).

2. Study design, study population and ethical issues

The study design is cross sectional, aiming to assess interaction between helminth and malaria infections. Ethical clearance was obtained from the regional ethic committee of Lambaréné (CERIL). Prior to enrolment, the protocol was explained to each participant or to their legal parent or guardian if they were under 18 years of age and a written informed consent was obtained. The study was preceded by a pilot phase so as to set up the study procedures and better characterize the study area and the study population. To be eligible for the study, subjects had to be between 5 and 50 years of age and living in the study area for at least one year. We did not include subjects with a hemoglobin level below 8 g/dl or with known HIV infection. In addition, intake of praziquantel less than one year prior to the study was also a non-inclusion criterion.

3. Recruitment of participants, data and samples collection

Local authorities and villagers were informed about the study through meetings and home visits. A parasitological laboratory was set up in the Bindo area by the study team in order to process the samples on time during the study. The relative proximity of the Pk15 area to Lambaréné meant that samples for parasitological diagnosis could be processed in the main laboratory in Lambaréné.

After informing the communities, all the houses in both villages were identified and geographical coordinates were taken to allow mapping of the study area by global positioning system (GPS). Villagers were then visited at home by field workers. During these visits the study was explained in detail to the occupants, questions regarding the study were answered and people willing to participate and who were eligible were enrolled.

Following the informed consent process a questionnaire was administered to each participant or their parents in case they were unable to answer, to collect demographic and health history data. For

all eligible participants a clinical examination was performed to determine the presence of hepatomegaly and/or splenomegaly as well as other relevant health conditions, urine samples were obtained for detection of *S. haematobium* infection and blood was drawn for parasitological and immunological analysis as described in sections below. All the data collected during the study was on paper forms which were then entered into OpenClinical, clinical trial software for electronic data capture.

4. Parasitological examination

4.1. Detection of Schistosoma haematobium eggs

Presence of *S. haematobium* eggs was detected microscopically in 10 ml of fresh urine passed through a filter of 12-mm pore-size (Millipore).

A subject was classified as free of infection if no *S. haematobium* egg was detected in three samples of urine collected in three consecutive days. Any subject with at least one *S. haematobium* egg found in the urine was classified as infected.

4.2. Detection of Plasmodium spp. blood stage parasite

Plasmodium spp. infection was assessed by microscopic examination. Presence of asexual form of the parasite will be determined using the Lambaréné method as described elsewhere (Kremsner et al. 1988; Planche et al. 2001). On the other hand presence of sexual form of the parasite will be established by the WHO method after a minimum count of 1000 white blood cells.

Real time-PCR will be performed on DNA extracted from EDTA blood pellet kept frozen at minus 80°C. This will be done in addition to microscopy examination to increase the sensitivity of parasites detection. A detailed description of the procedure has been published elsewhere (Adegnika et al. 2006).

4.3. Detection of microfilaria in blood

Microfilaria species in blood will be detected by leucoconcentration and microscopy using a modified Knott's technique (Goldsmid 1970). One milliliter of blood will be collected in an EDTA tube and dispensed in an equal volume of 2% saponin lysing solution. After centrifugation the sediment will be transferred to a slide. The entire slide will be examined using a microscope and the number of microfilaria will be counted. Differentiation between *Loa loa* and *Mansonela spp*. will be based on the identification of the sheath of *Loa loa* after the addition of one drop of methylene blue to the slide.

5. Immunological analysis

For immunological assays blood will be drawn in the field from the participants and will be brought to the laboratory facilities within 6 hours. In order to address the research questions immunological assays including antibody measurements by ELISA, whole blood culture assays, human peripheral mononuclear cells (PBMC) isolation and stimulations, cytokine production analysis by flow cytometry (through intracellular staining) and multiplex bead analysis (released cytokines in supernatants measured by luminex), and gene expression by multiplex analysis will be performed as described below.

5.1. Humoral immunological assays

We will measure total IgG specific to asexual and sexual forms of *P. falciparum* by ELISA as previously described (Ouédraogo et al. 2011). Antibody response to the apical membrane antigen 1 (AMA-1), merozoite surface protein 1_{19} (MSP-1_{19}) and glutamate rich protein (GLURP) antigens will be used as a markers of cumulative exposure to *P. falciparum*. On the other hand total IgG to Pfs48/45 and Pfs230 will be measured to determine *P. falciparum* gametocyte carriage and antibody response over time as described earlier (Bousema et al. 2006).

5.2. Cellular immunological assays

5.2.1. Media preparation

For the immunological essays media will be prepared as described in below.

RPMI-S1 medium: RPMI-1640 (Invitrogen, Breda, The Netherlands) supplemented with 100 U/ml penicillin (Astellas Pharma B.V.), 100 μg/ml streptomycin (Sigma-Aldrich, Zwijndrecht, The Netherlands), 1 mM pyruvate (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 2 mM L-glutamine (Sigma-Aldrich, Zwijndrecht, The Netherlands).

RPMI-S2 medium: RPMI-1640 (Invitrogen, Breda, The Netherlands) supplemented with 20% Fetal Calf Serum (FCS, Greiner Bio-One, Alphen a/d Rijn, The Netherlands), , 100 U/ml penicillin and 100 μg/ml streptomycin

RPMI-S3 medium: RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin

IMDM-S1 medium: IMDM (Invitrogen, Breda, The Netherlands) supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate and 2 mM L-glutamine).

IMDM-S2 medium: IMDM supplemented with 10% FCS,100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate and 2 mM L-glutamine

Freezing medium: RPMI-1640 + 20%FCS supplemented with 20% Dimethyl sulfoxide (DMSO, Merck KGaA, Darmstadt, Germany)

FACS buffer: 500 ml PBS (Invitrogen, Breda, The Netherlands), 0.5% BSA (Roche Diagnostics GmbH, Mannheim, Germany), 2 mM EDTA (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 2 ml of 0.5 M stock).

FACS staining solution: FACS buffer with 1% human FcgR-binding inhibitor (eBioscience, San Diego, CA, USA)

Permeabilization buffer: FACS buffer with 0.5% saponin (Sigma-Aldrich, Zwijndrecht, The Netherlands)

5.2.2. Whole blood culture

Heparinized blood will be diluted in equal volume of RPMI-S1 medium and cultured in 96 wells round bottom plates. Hundred micro liters of diluted blood will be distributed in each well and cultured with 100 µl of RPMI-S1 medium and with one of the following stimuli: CPG (5 µg/ml, Cayla-Invivogene Europe, Toulouse, France), LPS (100 ngml, Cayla-Invivogene Europe, Toulouse, France), LPS + CPG, schistosoma eggs antigens (SEA, 10 µg/ml, prepared by the Leiden University Medical center, The Netherlands (LUMC)), adult worm antigen (AWA , 10 µg/ml, prepared by the Leiden University Medical center, The Netherlands (LUMC)), PAM3 (100 ng/ml, EMC Microcollection GmbH, Tübingen, Germany), FSL1 (100 ng/ml, Cayla-Invivogene Europe, Toulouse, France), CLO97 (1 µg/ml, Cayla-Invivogene Europe, Toulouse, France). After 24 hours incubation at 37°C, supernatants will be collected, split into 2 tubes and kept at −20°C until further analysis.

5.2.3. PBMC isolation

For this assay, peripheral blood will be collected in sodium heparinized tubes (BD, Franklin Lakes, NJ, USA). PBMC will be isolated from blood by density gradient centrifugation on Ficoll (Apotheek AZL, Leiden, The Netherlands) as described earlier (Yazdanbakhsh et al. 1993). We expect an average of 25 x 10^6 PBMCs per donor, which will be used for the different assays planned, as follows in sections below.

5.2.4. Cryopreservation of PBMC

PBMC will be resuspended at the concentration of 10 x 10^6 in a solution of RPMI-S2 without glutamax. An equal volume of a freezing medium will then be added to the cell suspension. Finally the total amount of cells will be split into cryovials. One milliliter of the cells suspension containing 5 x 10^6 PBMC will be transferred in each cryovial. All the cryovials will be put in a Mr Frosty and will be kept

overnight in a -80^{o} freezer and transferred into a liquid nitrogen containing tank the next day for long term storage.

5.2.5. Thawing and resting of cryopreserved cells

Cryovials will be collected from the Liquid nitrogen tank and defrosted in a 37^{o} C water bath. For each cryovial the cell suspension is transferred into a corresponding 50 ml conical tube. PBMC will then be washed two times with RPMI-S3 without glutamax and resuspended at the concentration of 0.5 to 2 x 10^{6} cells/ml in RPMI-S3 medium. PBMC will finally be allowed to rest for 4–6 hour in a 5% CO_2 incubator at 37^{o} C.

5.2.6. PMA/Ionomycin stimulation of PBMC for intracellular cytokine measurement

For this assay 3.5 x 10^{6} PBMC will be transferred to a 5 ml tube and washed with 2 ml of IMDM-S2 medium. The suspension will be spun for 5 minutes at 20°C, 1500 rpm. Supernatant will then be discarded and the cells will be resuspended in 300 µl of IMDM-S1. Hundred micro liter of the cells suspension (1x10^{6}) will be cultured in 96 wells round bottom plate with PMA/Ionomycin (100 ng/ml and 1 µg/ml respectively, Sigma Aldrich), SEB (10 µg/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands) or medium as negative control. After 2 hours incubations at 37°C, 4 µg/ml of Brefeldin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) will be added to each well and cells will be incubated for 4 additional hours at 37°C before fixation by 1.9% paraformaldehyde (PFA) fixative (Sigma-Aldrich, Zwijndrecht, The Netherlands).

5.2.7. PBMC depletion of $CD4^{+}CD25^{high}$ regulatory T cells

Depletion of PBMC from $CD4^{+}CD25^{high}$ regulatory T cells (Treg) will aim to assess how Treg cell function is affected during helminth and malaria coinfection. Total and Treg cell-depleted PBMC will be stimulated by different antigens or mitogens and their response will be compared. A total of 12 X 10^{6} PBMC will be needed for this experiment. Treg cells will be isolated by MACS using the

$CD4^+CD25^{high}$ regulatory T cells kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). As already described cells isolation will be done in a two-step procedure (Wammes et al. 2012). Briefly, $CD4^+$ cells will be enriched by negative selection and then labeled with $CD25^+$ microbeads for a subsequent selection of $CD4^+CD25^+$ regulatory T cells. It should be noted that both depleted and whole PBMC cell populations will undergo identical procedure involving the MACS columns but in the latter population, $CD4^+CD25^+$ regulatory T cells will be added back (mock depletion).

5.2.8. Proliferation assay of total and $CD4^+CD25^{high}$ regulatory T cells depleted PBMC

This assay will be performed using the green-fluorescent dye carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich, Zwijndrecht, The Netherlands) so as to follow cells proliferation (Quah et al. 2007). To load the cells with CFSE, total and depleted PBMC will be resuspended in PBS (Invitrogen, Breda, The Netherlands) at a concentration of 2 x 10^7 cells/ml. CFSE will then be added at the concentration of 2 µM and cells will be incubated for 15 minutes at room temperature in the dark. After the incubation time, CFSE staining will be stopped by adding 4 ml of IMDM-S2 medium for 1 minute. Finally, cells will be spun down for 5 min 1800 rpm 20°C and the supernatant will be decanted. The loaded cells will then be ready for culture.

5.2.9. Antigen stimulation of total and $CD4^+CD25^{high}$ regulatory T cells depleted PBMC

CFSE-labeled Treg cell-depleted and undepleted PBMC, will be cultured in a 96 wells round bottom plates. Cells will be seeded at the concentration of 4 X 10^5 cells and cultured for 3 days with 100 µl of malaria infected red blood cells (iRBC; 1 x 10^6 cells per well prepared by the Leiden University Medical center, The Netherlands (LUMC)), malaria uninfected red blood cells (uRBC; 1 x 10^6 cells per well prepared by the Leiden University Medical center, The Netherlands), SEB (10 µg/ml), PPD (10 µg/ml, Statens Serum Institute, Denmark, Copenhagen), SEA (20 µg/ml) , AWA (10 µg/ml). At day 3 of culture,

supernatants will be collected and keep at −20°C for cytokine measurements by luminex or ELISA. In addition, cells will be harvested, fixed with 1,9% PFA for 15 min and stored at −80°C until further analysis for cell division detected by CSFE labeling using flow cytometry.

5.3. Cytokine production analysis

Cytokine production will be measured in the supernatants obtained, after three days stimulation of total and $CD4^{+}CD25^{high}$ regulatory T cells depleted PBMC and 24 hours of whole blood culture. Cytokine levels will be measured using the multiplex beads array immunoassay or ELISA according to standard procedure. A quantification of different cytokines will be done in two different panels to characterize the cytokine profile involved in innate immunity (IFNa2, IL1b, IL6, IL10, IL12p70, IL-13, IL-23, IFNg, MCP1, MIP1a, MIP1b, TNFa and IP-10) and adaptive immunity (TNF-a, IFN-g, IL-2, IL-4, IL-5, IL-13, IL-17A, IL-17 F, IL-22, IL-10, and IL-21) respectively. The multiplex beads kit will be obtained from Bio-Rad Laboratories and samples will be acquired on the Bio-Plex 200 system (Bio-Rad Laboratories) following the manufacturer recommendations. The most informative cytokines will be used for measurement of further samples.

5.4. FACS analysis

5.4.1. Cell surface marker staining

Cells immunophenotyping will be performed by flow cytometry. Cells will be seeded at the concentration of 2 X 10^5 to 4X 10^5 cells per well in a 96 well FACS V-bottom plates and resuspended in FACS buffer. Extracellular staining of the cells will be done by a mixture of all the fluorescent labeled antibodies of interest prepared in a FACS staining solution at optimal working concentration. After centrifugation and discarding of FACS buffer, cells will be resuspended in 30 µl of the antibody solution and incubated for 30 minutes in the dark at 4 ° C. Following this step, 100 µl of FACS buffer will be added to each well, cells will be spun again and supernatant discarded. Finally the labeled cells will be resuspended in 50 µl of FACS buffer for acquisition.

Acquisition will be performed on a FACS calibur flow cytometer (Becton Dickinson Biosciences BD) and data will be analysed by Flowjo software (Treestar Inc., Ashland, OR, USA). We will use different panels of fluorescently labeled antibodies and cluster various differentiation markers to be able to identify B cells, DCs, monocytes and different T cells subsets and other interesting cells of the immune system such as natural killer (NK) cells or γδ T cells that have recently been shown to be associated with immune response against malaria.

5.4.2. Intracellular staining

To allow the assessment of cell-specific cytokine production, intracellular cytokines in combination with various subset markers for dendritic cells (DCs), B cells, monocytes and/or (γδ) T cells will be labeled by fluorescent antibodies. Staining of intracellular cytokines will be performed by a two step procedure consisting of permeabilization and staining of the cells. For PFA fixed cells permeabilization will be performed using a permeabilization buffer. For intracellular staining of FoxP3, PBMC will be fixed and permeabilized by a FoxP3 fixation and permeabilization kit (eBioscience, San Diego, CA, USA). FACS staining will be done as described in the previous paragraph with one additional washing step with permeabilization buffer before adding the antibody mix.

5.5. Innate gene profiling

Gene profiling will be performed by Reverse Transcription Multiplex Ligation-dependent Probe Amplification (RT-MLPA) in a subset of the study participants. A volume of 2.5 ml of blood will be collected in a PAXgene Blood RNA tube (PreAnalytiX, Quiagen, Germany) per subject. Gene expression profiling of various pattern recognition receptors (PRRs) as well as several key cytokines and chemokines (CCL2; 5 and 22, CXCL13, IL-10, IL-12p40/35, IL-23p19) will be done as detailed previously (Joosten et al. 2012) to assess whether cells signaling and trafficking is differentially affected in single infection versus coinfection.

6. Analysis Plan

The aim of this project is a) to determine how concurrent schistosome infections can affect malaria infections and malaria transmission intensity. Specifically we will assess how levels antibodies to asexual and gametocyte antigens, marker of gametocytogenesis are influenced by concurrent schistosome infection and b) to assess how general and malaria-specific immune responses are modified by concurrent schistosome infection. By general we mean responses to mitogens and activation status of immune cells. On the other hand by malaria specific-immune response we refer to cytokine production and activation in response to infected red blood cells. This study will address the following research questions:

- What is the impact of *S. haematobium* infection on malaria transmission: how does it impact gametocyte carriage and humoral response to gametocytes?
- What is the level of pro-inflammatory and anti-inflammatory cytokines during malaria and/or schistosome infection in response to stimuli that activate the innate and adaptive immune system?
- Are the phenotype of T cells, B cells, DCs and monocytes different during malaria and/or schistosome infection in general, and in an antigen specific manner, in particular?
- What is the functional capacity of $CD4^{+}CD25^{high}$regulatory T cells, as assessed by depletion on the immune response in case of malaria and/or schistosome infection?
- Is the gene expression signature of the immune system altered during schistosoma and malaria coinfection?

These questions will be answered by considering the two study outcomes represented first by the infectious status of participants and secondly by the immune response. For this cross sectional study, four groups will be compared; Plasmodium spp. and S. haematobium uninfected , *Plasmodium spp.* and *S. haematobium* coinfected, *Plasmodium spp.* only infected as well as *S. haematobium* only infected group. Baseline characteristics will be determined and differences of cellular immune response between groups will be assessed.

7. Sample size calculation

For detailed immunological studies the sample size calculation was based on the proportion of responders between infected versus uninfected participants (either malaria or schistosomiasis infection). Considering previous unpublished data we hypothesized that the proportion of responders with regard to the levels of $CD4^{+}CD25^{high}FoxP3^{+}$ T cells will be 15% in the uninfected group and 50% in the infected group necessitating a sample size of 28 subjects per group (power = 0.80 and p = 0.05). To correct for an estimated 10% failure rate in sample processing we included 31 subjects per group. This sample size will permit to measure other outcomes like difference in cytokine production with adequate power. For example the study will have a power of 0.7 to see an increase in IL10 responders from 30 to 60%, which we expect from schistosomiasis carriers (Hartgers et al. 2009; Meurs et al. 2011; van der Kleij et al. 2004).

On the other hand the above calculated sample size only gives a power of 0.32 to detect an effect of helminth infection on antibody response to sexual or asexual stage antigens. Therefore we run an additional sample size calculation that was based on data gathered from an immuno epidemiological study conducted in a setting similar to our study site. From this study it appears that gametocyte antigens usually elicit a weaker antibody response than asexual stage antigens and that the percentage of IgG responders to Pfs48/45 and Pfs230 was around 22 to 28% (Ouédraogo et al. 2011). Given the fact that we did not have data comparing antibody response specific to Pfs48/45 and Pfs230 between schistosoma infected and uninfected subjects, we hypothesize that helminth infection will lead to a maximum twofold change in antibody response to Pfs48/45 and Pfs230. Considering this we calculated that a sample size of 63 subjects per group (power = 0.90 and p = 0.05) would be adequate to answer our research question. Based on the literature it antibody response to sexual or asexual stage antigens may vary according to the age of the subjects. Hence as our study will include both children and adult our final sample size will be of 126 subjects per group.

8.Results from pilot study

Pilot studies have been conducted in the PK15 area and the Bindo village to characterize the study population and to set up the study procedure. All houses in both areas have been identified and their GPS coordinates recorded (Figure 2).

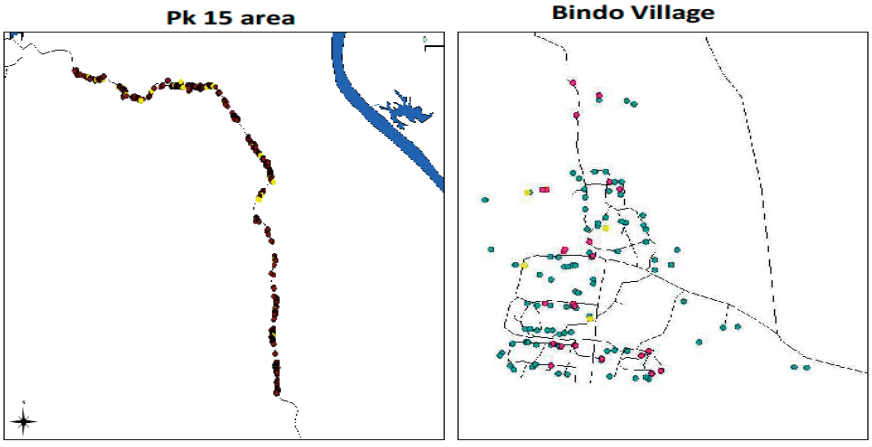

Figure 2: Mapping of the PK15 area and the Bindo village. Each dot represents a single house. In the left panel the yellow dots indicate houses where Schistosoma haematobium infected subjects have been found during the screening phase. In the second panel the green dots represent houses not selected for the screening phase whereas the yellow and the red dots respectively represent houses where Schistosoma infected and uninfected subjects were living.

To obtain demographic data and to establish the epidemiological feature of *S. haematobium* infection in the study area a random selection of houses has been screened to represent around 10% of the population. Typical to low income countries the age pyramid curve presented a broad base as shown in Figure 3. In both areas the age pyramids show that only few adults from 20 to 49 are living in the village. This could be due to the migration of young adults to big cities for work or for study purposes. In Bindo this is less apparent as inhabitants are employed by the palm oil company.

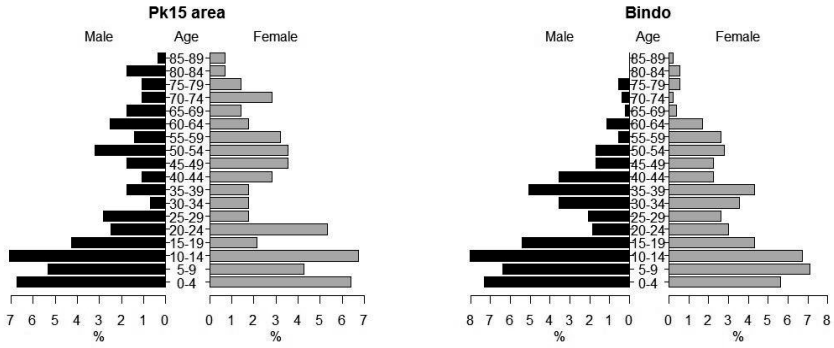

Figure 3: Age pyramids of the population of the Pk15 area and the Bindo village.

Regarding the *S. haematobium* infection we found an overall prevalence of 43% in the PK15 area and 15% in Bindo village. This difference between the two areas could be explained by the fact that in the Pk15 area streams represent the first source of water compared to the Bindo village where public water pumps are available. As represented in the age prevalence curve, children were the most infected by *S. haematobium* and showed the highest infection intensity (Figures 4 and 5).

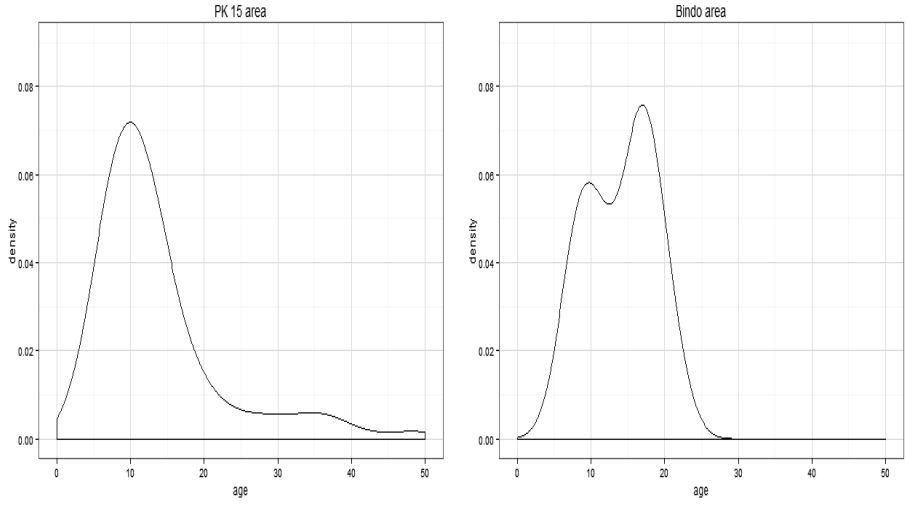

Figure 4: Kernel density estimation of *Schistosoma haematobium* infection per age in the Pk15 area and the Bindo village. This estimation is based on results of a random sample of around 10% of the population of both area.

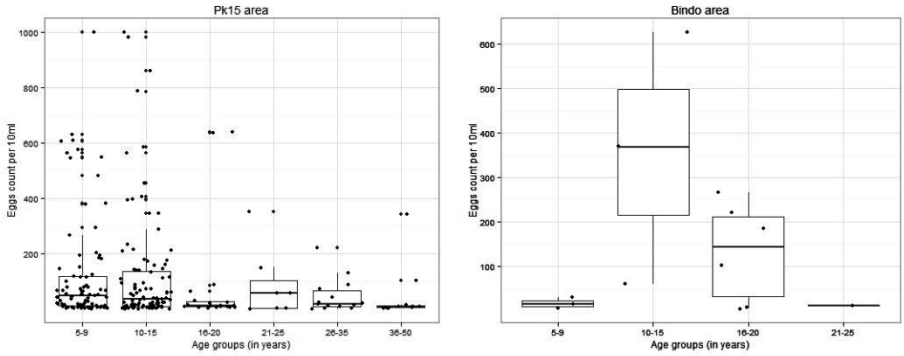

Figure 5 Intensity of *Schistosoma haematobium* infection per age group in the different study area. Each dot represents the eggs count of a single subject.

Conclusion

Polyparasitism is usually described as common in tropical and sub tropical areas where the majority of the population is simultaneously exposed to various pathogens. Malaria and helminth coinfection is very common and it is important to understand their interaction. So far only few studies have been conducted on the interaction between these two pathogens and their effect on the human host in much detail. Moreover, the data generated so far provide contradictory results. At the immunological level, the data available has shown that chronic helminthiasis can modulate and impair immune responses specific to malaria antigens. It has been proposed that modulation may rely on two mechanisms. Firstly, through the skewing of cytokine production toward a Th2, which could alter the antibody isotypes and cellular responses generated against malaria parasites. Secondly, by the induction of regulatory cells which indue a hypo responsiveness milieu impairing the cellular response against *Plasmodium*. Despite this general assumption that helminth infections modulate malaria immunity, many questions need to be answered in great detail to understand and unequivocally establish whether there is an interaction between these two parasites. One of them concerns the effect of chronic helminth infection on cell specific immune responses in terms of $CD4^{+}CD25^{high}$ Treg cells and the control of other cytokine producing effector (T) cells. What is new in this study is the aim to unravel general patterns in innate and adaptive immune responses both at the

cellular and molecular level, providing insight in innate immunity shifts that precede and dictate adaptive immune responses in single or co-infected individuals. The identification of malaria responsive cell subsets is needed; the contribution of DCs, monocytes, CD4 or CD8 T cells, γδ T cells or NK cells to malaria antigen-specific responses and the influence of helminth infections on these subsets will be studied.

These studies will shed light on the possible interaction between schistosome and malaria parasites and pave the way for future interventional studies.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

UAN, JFZ, RFKK, ENF, JH, GMN, ALBH, JCDA carried on the study on the field. They were responsible of the screening, the enrolment and the follow up of the study participants. HKM and AMN performed the diagnosis of parasitic infection. UAN, MML, HKM, AMN, LHM, LW, MM, YK carried on the different immunological assays. IS, BL and HS advise on the epidemiological aspect of the study. HS advise on the immunological aspect of the study. PGK, MY and AAA designed and coordinated the study.

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References

Adegnika AA, Kremsner PG (2012) Epidemiology of malaria and helminth interaction: a review from 2001 to 2011. Curr Opin HIV AIDS 7:221–224, 10.1097/COH.0b013e3283524d90

Adegnika AA, Verweij JJ, Agnandji ST, Chai SK, Breitling LP, Ramharter M, Frolich M, Issifou S, Kremsner PG, Yazdanbakhsh M (2006) Microscopic and Sub-Microscopic Plasmodium Falciparum Infection, but Not Inflammation Caused by Infection, Is Associated with Low Birth Weight. Am J Trop Med Hyg 75:798–803

Adegnika AA, Ramharter M, Agnandji ST, Ateba Ngoa U, Issifou S, Yazdanbahksh M, Kremsner PG (2010) Epidemiology of parasitic co-infections during pregnancy in Lambaréné, Gabon. Trop Med Int Health 15:1204–1209, 10.1111/j.1365-3156.2010.02598.x

Akue JP, Nkoghe D, Padilla C, Moussavou G, Moukana H, Mbou RA, Ollomo B, Leroy EM (2011) Epidemiology of concomitant infection due to Loa loa and Mansonella perstans in Gabon. PLoS Negl Trop Dis 5:e1329, 10.1371/journal.pntd.0001329

Belkaid Y (2007) Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol 7:875–888, 10.1038/nri2189

Boel M, Carrara VI, Rijken M, Proux S, Nacher M, Pimanpanarak M, Paw MK, Moo O, Gay H, Bailey W, Singhasivanon P, White NJ, Nosten F, McGready R (2010) Complex Interactions between Soil-Transmitted Helminths and Malaria in Pregnant Women on the Thai-Burmese Border. PLoS Negl Trop Dis 4:e887, 10.1371/journal.pntd.0000887

Bouharoun-Tayoun H, Druilhe P (1992) Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. Infect Immun 60:1473–1481

Bousema JT, Roeffen W, Kolk MVD, Vlas SJD, Vegte-Bolmer MVD, Bangs MJ, Teelen K, Kurniawan L, Maguire JD, Baird JK, Sauerwein RW (2006) Rapid Onset of Transmission-Reducing Antibodies in Javanese Migrants Exposed to Malaria in Papua, Indonesia. Am J Trop Med Hyg 74:425–431

Brooker S, Akhwale W, Pullan R, Estambale B, Clarke SE, Snow RW, Hotez PJ (2007) Epidemiology of plasmodium-helminth co-infection in Africa: populations at risk, potential impact on anemia, and prospects for combining control. Am J Trop Med Hyg 77:88–98

Courtin D, Djilali-Saïah A, Milet J, Soulard V, Gaye O, Migot-Nabias F, Sauerwein R, Garcia A, Luty AJF (2011) Schistosoma haematobium infection affects Plasmodium falciparum-specific IgG responses associated with protection against malaria. Parasite Immunol 33:124–131, 10.1111/j.1365-3024.2010.01267.x

Diallo TO, Remoue F, Schacht AM, Charrier N, Dompnier J-P, Pillet S, Garraud O, N'diaye AA, Capron A, Capron M, Riveau G (2004) Schistosomiasis co-infection in humans influences inflammatory markers in uncomplicated Plasmodium falciparum malaria. Parasite Immunol 26:365–369, 10.1111/j.0141-9838.2004.00719.x

Goldsmid JM (1970) Studies on the laboratory diagnosis of human filariasis: preliminary communication. J Clin Pathol 23:632–635

Griffiths EC, Pedersen AB, Fenton A, Petchey OL (2011) The nature and consequences of coinfection in humans. J Infect 63:200–206, 10.1016/j.jinf.2011.06.005

Hartgers FC, Yazdanbakhsh M (2006) Co-infection of helminths and malaria: modulation of the immune responses to malaria. Parasite Immunol 28:497–506, 10.1111/j.1365-3024.2006.00901.x

Hartgers FC, Obeng BB, Kruize YCM, Dijkhuis A, McCall M, Sauerwein RW, Luty AJF, Boakye DA, Yazdanbakhsh M (2009) Responses to malarial antigens are altered in helminth-infected children. J Infect Dis 199:1528–1535, 10.1086/598687

Hotez PJ, Kamath A (2009) Neglected tropical diseases in sub-saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis 3:e412, 10.1371/journal.pntd.0000412

Joosten SA, Goeman JJ, Sutherland JS, Opmeer L, de Boer KG, Jacobsen M, Kaufmann SHE, Finos L, Magis-Escurra C, Ota MOC, Ottenhoff THM, Haks MC (2012) Identification of biomarkers for tuberculosis disease using a novel dual-color RT-MLPA assay. Genes Immun 13:71–82, 10.1038/gene.2011.64

Kremsner PG, Zotter GM, Feldmeier H, Graninger W, Rocha RM, Wiedermann G (1988) A comparative trial of three regimens for treating uncomplicated falciparum malaria in Acre, Brazil. J Infect Dis 158:1368–1371

Langhorne J, Cross C, Seixas E, Li C, von der Weid T (1998) A role for B cells in the development of T cell helper function in a malaria infection in mice. Proc Natl Acad Sci U S A 95:1730–1734

Le Hesran J-Y, Akiana J, Ndiaye EHM, Dia M, Senghor P, Konate L (2004) Severe malaria attack is associated with high prevalence of Ascaris lumbricoides infection among children in rural Senegal. Trans R Soc Trop Med Hyg 98:397–399, 10.1016/j.trstmh.2003.10.009

Lemaitre M, Watier L, Briand V, Garcia A, Le Hesran JY, Cot M (2014) Coinfection with Plasmodium falciparum and Schistosoma haematobium: additional evidence of the protective effect of Schistosomiasis on malaria in Senegalese children. Am J Trop Med Hyg 90:329–334, 10.4269/ajtmh.12-0431

Leoratti FMS, Durlacher RR, Lacerda MVG, Alecrim MG, Ferreira AW, Sanchez MCA, Moraes SL (2008) Pattern of humoral immune response to Plasmodium falciparum blood stages in individuals

presenting different clinical expressions of malaria. Malar J 7:186, 10.1186/1475-2875-7-186

Lyke KE, Dabo A, Arama C, Daou M, Diarra I, Wang A, Plowe CV, Doumbo OK, Sztein MB (2012) Reduced T regulatory cell response during acute Plasmodium falciparum infection in Malian children co-infected with Schistosoma haematobium. PloS One 7:e31647, 10.1371/journal.pone.0031647

Maizels RM, Smith KA (2011) Regulatory T cells in infection. Adv Immunol 112:73–136, 10.1016/B978-0-12-387827-4.00003-6

Maizels RM, Yazdanbakhsh M (2003) Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol 3:733–744, 10.1038/nri1183

Meurs L, Labuda L, Amoah AS, Mbow M, Ngoa UA, Boakye DA, Mboup S, Dièye TN, Mountford AP, Turner JD, Kremsner PG, Polman K, Yazdanbakhsh M, Adegnika AA (2011) Enhanced pro-inflammatory cytokine responses following Toll-like-receptor ligation in Schistosoma haematobium-infected schoolchildren from rural Gabon. PloS One 6:e24393, 10.1371/journal.pone.0024393

Mwangi TW, Bethony JM, Brooker S (2006) Malaria and helminth interactions in humans: an epidemiological viewpoint. Ann Trop Med Parasitol 100:551–570, 10.1179/136485906X118468

Nacher M (2011) Interactions between worms and malaria: good worms or bad worms? Malar J 10:259, 10.1186/1475-2875-10-259

Nacher M, Gay F, Singhasivanon P, Krudsood S, Treeprasertsuk S, Mazier D, Vouldoukis I, Looareesuwan S (2000) Ascaris lumbricoides infection is associated with protection from cerebral malaria. Parasite Immunol 22:107–113

Nacher M, Singhasivanon P, Silachamroon U, Treeprasertsu S, Krudsood S, Gay F, Mazier D, Looareesuwan S (2001) Association of helminth infections with increased gametocyte carriage during mild falciparum malaria in Thailand. Am J Trop Med Hyg 65:644–647

Noland GS, Graczyk TK, Fried B, Kumar N (2007) Enhanced Malaria Parasite Transmission from Helminth Co-Infected Mice. Am J Trop Med Hyg 76:1052–1056

Ouédraogo AL, Roeffen W, Luty AJF, de Vlas SJ, Nebie I, Ilboudo-Sanogo E, Cuzin-Ouattara N, Teleen K, Tiono AB, Sirima SB, Verhave J-P, Bousema T, Sauerwein R (2011) Naturally acquired immune responses to Plasmodium falciparum sexual stage antigens Pfs48/45 and Pfs230 in an area of seasonal transmission. Infect Immun 79:4957–4964, 10.1128/IAI.05288-11

Planche T, Krishna S, Kombila M, Engel K, Faucher JF, Ngou-Milama E, Kremsner PG (2001) Comparison of Methods for the Rapid Laboratory Assessment of Children with Malaria. Am J Trop Med Hyg 65:599–602

Quah BJC, Warren HS, Parish CR (2007) Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. Nat Protoc 2:2049–2056, 10.1038/nprot.2007.296

Raso G, Luginbühl A, Adjoua CA, Tian-Bi NT, Silué KD, Matthys B, Vounatsou P, Wang Y, Dumas M-E, Holmes E, Singer BH, Tanner M, N'goran EK, Utzinger J (2004) Multiple parasite infections and their relationship to self-reported morbidity in a community of rural Côte d'Ivoire. Int J Epidemiol 33:1092–1102, 10.1093/ije/dyh241

Remoue F, Diallo TO, Angeli V, Hervé M, de Clercq D, Schacht AM, Charrier N, Capron M, Vercruysse J, Ly A, Capron A, Riveau G (2003) Malaria co-infection in children influences antibody response to schistosome antigens and inflammatory markers associated with morbidity. Trans R Soc Trop Med Hyg 97:361–364

Sangweme DT, Midzi N, Zinyowera-Mutapuri S, Mduluza T, Diener-West M, Kumar N (2010) Impact of schistosome infection on Plasmodium falciparum Malariometric indices and immune correlates in school age children in Burma Valley, Zimbabwe. PLoS Negl Trop Dis 4:e882, 10.1371/journal.pntd.0000882

Simoonga C, Utzinger J, Brooker S, Vounatsou P, Appleton Cc, Stensgaard As, Olsen A, Kristensen Tk (2009) Remote sensing, geographical information system and spatial analysis for schistosomiasis epidemiology and ecology in Africa. Parasitology 136:1683–1693, 10.1017/S0031182009006222

Van der Kleij D, van den Biggelaar AHJ, Kruize YCM, Retra K, Fillie Y, Schmitz M, Kremsner PG, Tielens AGM, Yazdanbakhsh M (2004) Responses to Toll-like receptor ligands in children living in areas where schistosome infections are endemic. J Infect Dis 189:1044–1051, 10.1086/382089

Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, Smits HH, Supali T, Yazdanbakhsh M (2012) Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis 6:e1655, 10.1371/journal.pntd.0001655

Wildling E, Winkler S, Kremsner PG, Brandts C, Jenne L, Wernsdorfer WH (1995) Malaria epidemiology in the province of Moyen Ogoov, Gabon. Trop Med Parasitol 46:77–82

Yazdanbakhsh M, Paxton WA, Kruize YC, Sartono E, Kurniawan A, van het Wout A, Selkirk ME, Partono F, Maizels RM (1993) T cell responsiveness correlates differentially with antibody isotype levels in clinical and asymptomatic filariasis. J Infect Dis 167:925–931

Chapter 3

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

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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 naive 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 naive 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.

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 naive 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.

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 naive 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, naive 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 naive 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.

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 # $p< 0.1$.

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.

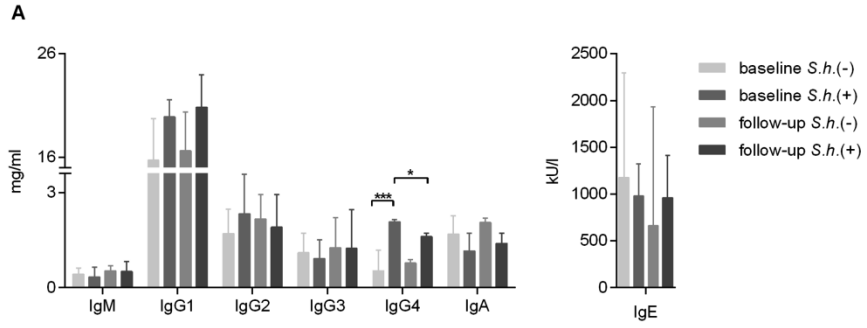

Figure 1. ***Serum immunoglobulin analysis.*** Serum samples were analyzed for total human IgM, IgG1, IgG2, IgG3, IgG4, IgA by Luminex and IgE by ELISA. Bars represent median with interquartile range. Number of donors in each group: baseline *S.h.* –ve n = 9, baseline *S.h.* +ve n = 10, follow-up *S.h.*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

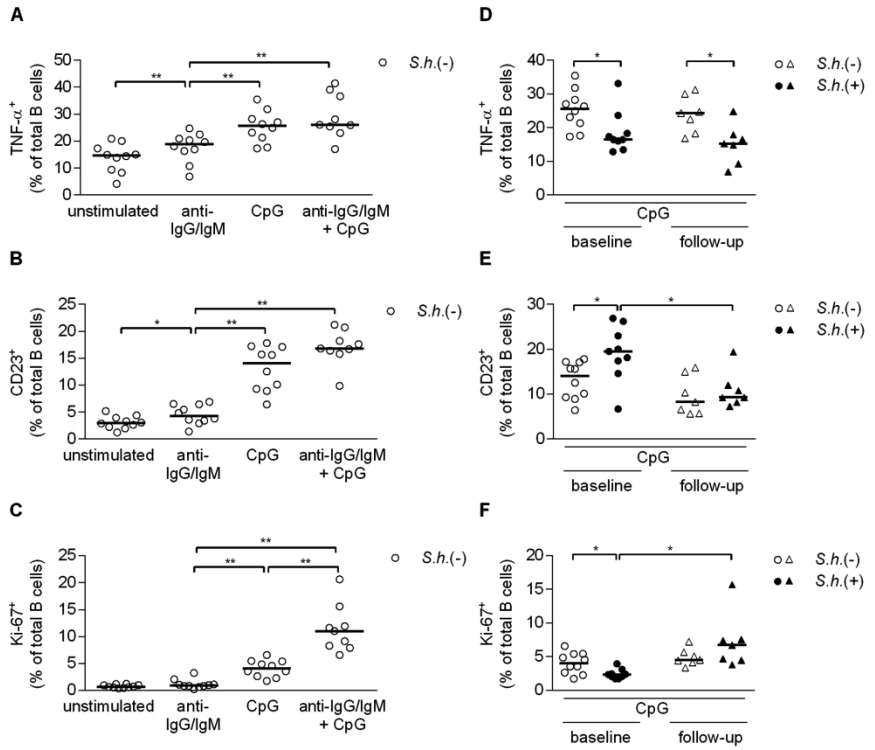

Figure 2. ***B cell inflammatory cytokine response, activation and proliferation.*** Total peripheral blood B cells were cultured with anti-IgG/IgM (2.5 μg/ml), CpG (5 μg/ml) or anti-IgG/IgM plus CpG for two days, restimulated with PMA/Ionomycin/LPS and BrefA and fixed. Levels of intracellular TNF-α (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.

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.

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 naive 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 naive B cells ($p = 0.062$) (Figure 3E) in schistosome-infected children and following treatment the level of naive 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 naive B cells (Spearman $r = -0.392$, $p < 0.05$).

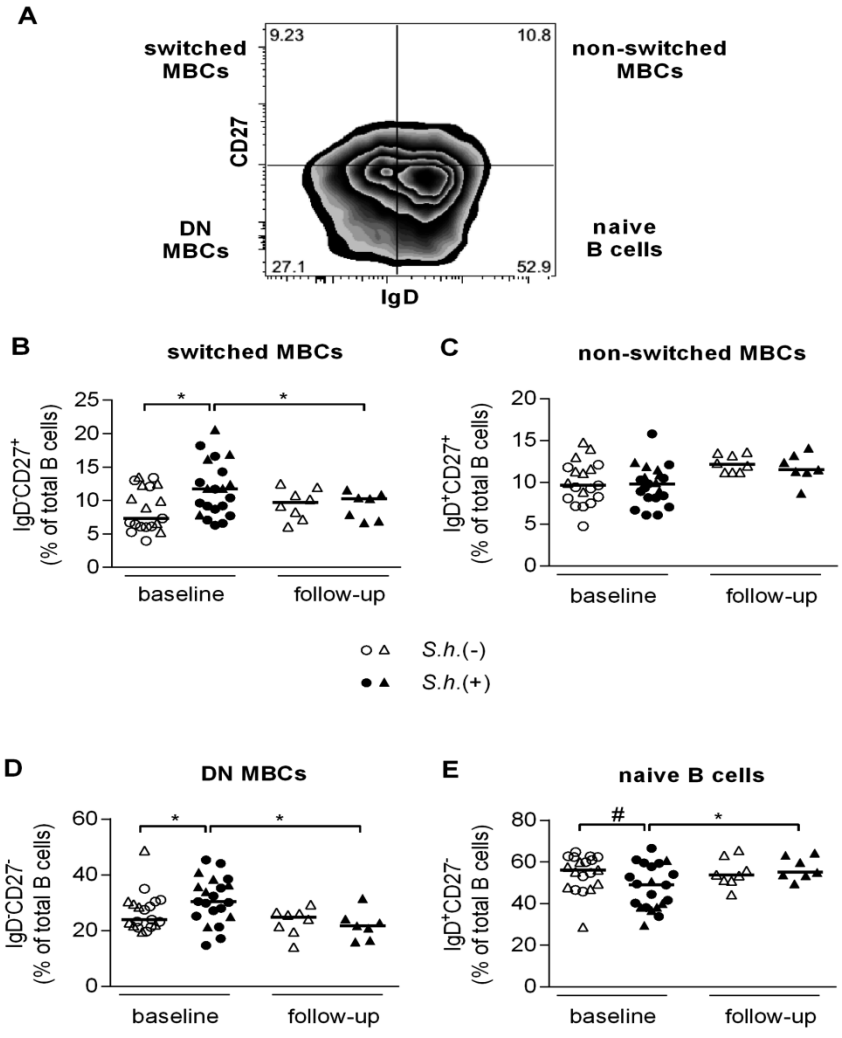

Figure 3. ***MBC analysis.*** PBMC were fixed and stained with B cell phenotyping markers (CD19, CD27 and IgD) and analyzed for B cell subsets by flow cytometry. B cell subset analysis was performed as shown in (A) (representative *S. haematobium*-uninfected child). Proportion of CD19-gated cells that were $CD27^{+}IgD^{-}$ (B, switched MBC), $CD27^{+}IgD^{+}$ (C, non-switched MBC), $CD27^{-}IgD^{-}$ (D, double negative MBC), and $CD27^{-}IgD^{+}$ (E, naive 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.

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 naive 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 naive 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) naive 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 naive 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 children, 2.7% and 2.8% respectively (p = 0.798).

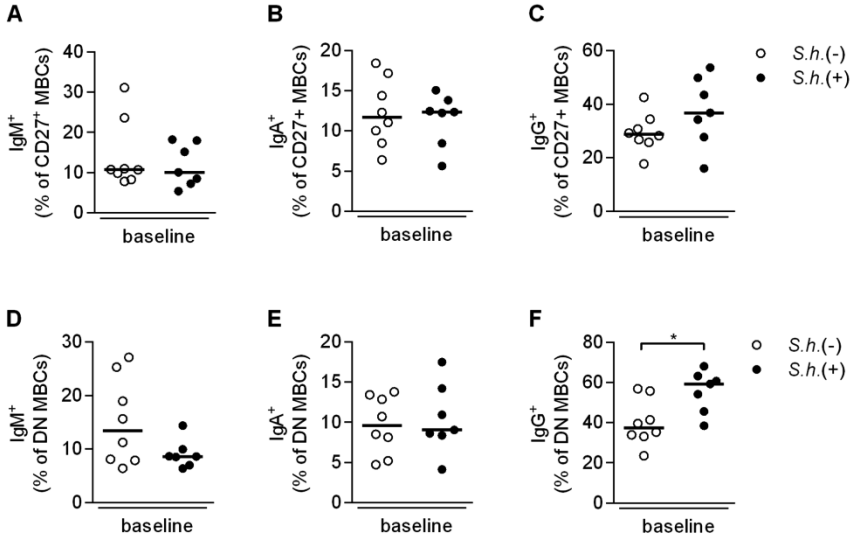

Figure 4. ***Expression of IgM, IgA and IgG on CD27$^+$ and DN MBCs.*** PBMC were fixed and expression of IgM, IgA and IgG on CD27$^+$ B cells was evaluated [46]. Proportions of CD19$^+$ gated cells that were IgM$^+$IgD$^-$CD27$^+$ (A), IgA$^+$IgM$^-$IgD$^-$CD27$^+$ (B), and IgG$^+$IgM$^-$IgD$^-$CD27$^+$ (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.

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

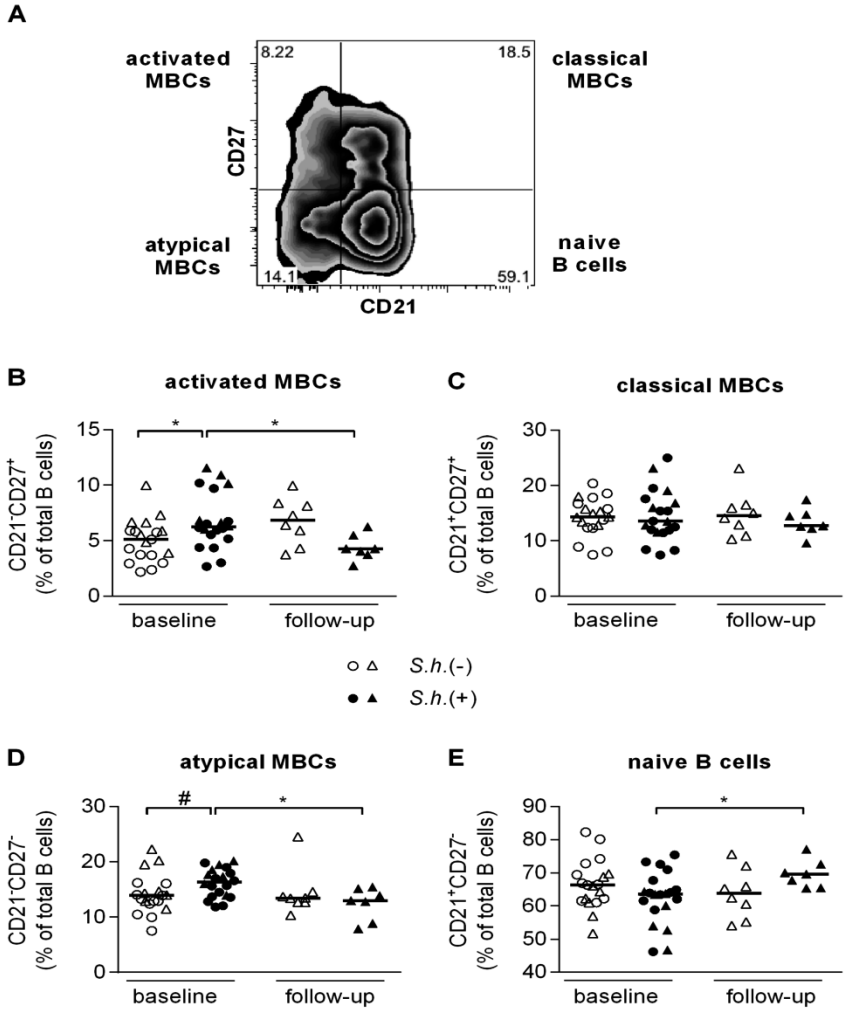

Figure 5. ***Atypical MBC analysis.*** PBMC were fixed and stained with B cell phenotyping markers (CD19, CD21 and CD27) and analyzed for B cell subsets by flow cytometry. B cell subset analysis was performed as shown in (A) (representative *S. haematobium*-uninfected child). Proportion of CD19-gated cells that were $CD27^{+}CD21^{-}$ (B, activated MBC), $CD27^{+}CD21^{+}$ (C, classical MBC), $CD27^{-}CD21^{-}$ (D, atypical MBC), and $CD27^{-}CD21^{+}$ (E, naive 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.

MBCs compared to classical MBCs ($CD27^-CD21^-$) and naive B cells ($CD27^-CD21^+$) in uninfected children (Figure S3A). Furthermore, HLA-DR expression was significantly higher on atypical MBCs and naive 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 naive B cells.

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 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 naive 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 $TNF\text{-}\alpha^+$ activated MBCs (Figure 6B) and classical MBCs (Figure 6C) and a trend for lower levels in the naive 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.

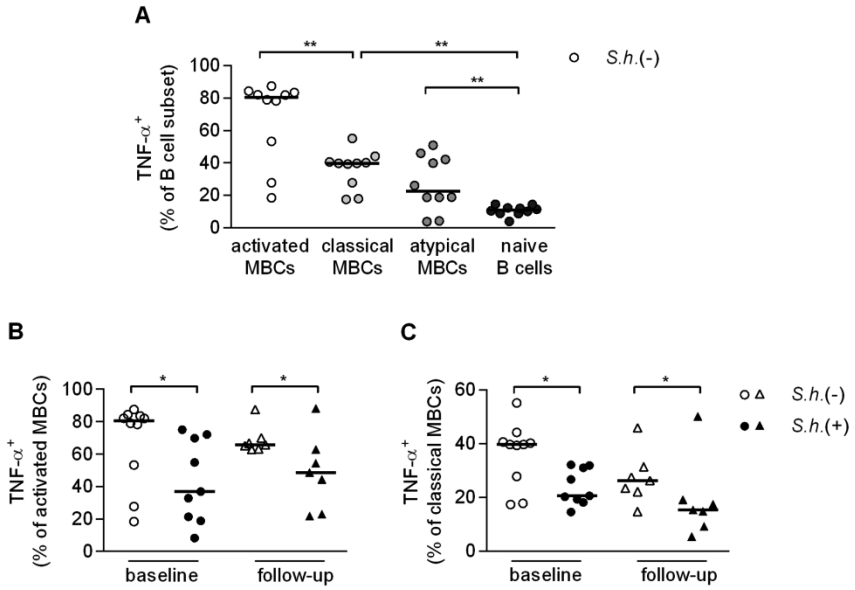

Figure 6. ***B cell subset inflammatory cytokine response, activation and proliferation.*** Total peripheral blood B cells were cultured with anti-IgG/IgM (2.5 μg/ml) for two days, restimulated with PMA/Ionomycin/LPS and BrefA, fixed and stained with B cell subset markers (CD10, CD19, CD21 and CD27) 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 naive 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 naive B cells following treatment is due to *de novo* generation of B cells from the bone marrow, expansion of the pre-existing naive 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 naive 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*.

Acknowledgements

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REFERENCES

1. WHO website (2012) Schistosomiasis. Available: http://www.who.int/mediacentre/factsheets/fs115/en/index.html. Accessed 26 June 2012.

2. Burke ML, Jones MK, Gobert GN, Li YS, Ellis MK, et al. (2009) Immunopathogenesis of human schistosomiasis. Parasite Immunol 31: 163-176.

3. van Riet E, Hartgers FC, Yazdanbakhsh M (2007) Chronic helminth infections induce immunomodulation: consequences and mechanisms. Immunobiology 212: 475-490.

4. Allen JE, Maizels RM (2011) Diversity and dialogue in immunity to helminths. Nat Rev Immunol 11: 375-388.

5. Woolhouse ME, Taylor P, Matanhire D, Chandiwana SK (1991) Acquired immunity and epidemiology of Schistosoma haematobium. Nature 351: 757-759.

6. Yazdanbakhsh M, Sacks DL (2010) Why does immunity to parasites take so long to develop? Nat Rev Immunol 10: 80-81.

7. Jankovic D, Wynn TA, Kullberg MC, Hieny S, Caspar P, et al. (1999) Optimal vaccination against Schistosoma mansoni requires the induction of both B cell- and IFN-gamma-dependent effector mechanisms. J Immunol 162: 345-351.

8. Sher A, Smithers SR, Mackenzie P (1975) Passive transfer of acquired resistance to Schistosoma mansoni in laboratory mice. Parasitology 70 Part 3: 347-357.

9. Mangold BL, Dean DA (1986) Passive transfer with serum and IgG antibodies of irradiated cercaria-induced resistance against Schistosoma mansoni in mice. J Immunol 136: 2644-2648.

10. Vereecken K, Naus CW, Polman K, Scott JT, Diop M, et al. (2007) Associations between specific antibody responses and resistance to reinfection in a Senegalese population recently exposed to Schistosoma mansoni. Trop Med Int Health 12: 431-444.

11. Khalife J, Dunne DW, Richardson BA, Mazza G, Thorne KJ, et al. (1989) Functional role of human IgG subclasses in eosinophil-mediated killing of schistosomula of Schistosoma mansoni. J Immunol 142: 4422-4427.

12. McManus DP, Loukas A (2008) Current status of vaccines for schistosomiasis. Clin Microbiol Rev 21: 225-242.

13. Pene J, Rousset F, Briere F, Chretien I, Bonnefoy JY, et al. (1988) IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. Proc Natl Acad Sci U S A 85: 6880-6884.

14. Mwinzi PN, Ganley-Leal L, Black CL, Secor WE, Karanja DM, et al. (2009) Circulating CD23+ B cell subset correlates with the development of resistance to Schistosoma mansoni reinfection in occupationally exposed adults who have undergone multiple treatments. J Infect Dis 199: 272-279.

15. Black CL, Muok EM, Mwinzi PN, Carter JM, Karanja DM, et al. (2010) Increases in levels of schistosome-specific immunoglobulin E and CD23(+) B cells in a cohort of Kenyan

children undergoing repeated treatment and reinfection with Schistosoma mansoni. J Infect Dis 202: 399-405.

16. Harris N, Gause WC (2011) To B or not to B: B cells and the Th2-type immune response to helminths. Trends Immunol 32: 80-88.

17. van der Vlugt LE, Labuda LA, Ozir-Fazalalikhan A, Lievers E, Gloudemans AK, et al. (2012) Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. PLoS One 7: e30883.

18. Crotty S, Ahmed R (2004) Immunological memory in humans. Semin Immunol 16: 197-203.

19. Agematsu K, Nagumo H, Yang FC, Nakazawa T, Fukushima K, et al. (1997) B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. Eur J Immunol 27: 2073-2079.

20. Klein U, Rajewsky K, Kuppers R (1998) Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med 188: 1679-1689.

21. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, et al. (2008) Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. J Exp Med 205: 1797-1805.

22. Moir S, Fauci AS (2009) B cells in HIV infection and disease. Nat Rev Immunol 9: 235-245.

23. Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, et al. (2009) Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. J Immunol 183: 2176-2182.

24. Weiss GE, Clark EH, Li S, Traore B, Kayentao K, et al. (2011) A positive correlation between atypical memory B cells and

Plasmodium falciparum transmission intensity in cross-sectional studies in Peru and Mali. PLoS One 6: e15983.

25. Ehrhardt GR, Hsu JT, Gartland L, Leu CM, Zhang S, et al. (2005) Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. J Exp Med 202: 783-791.

26. Ehrhardt GR, Hijikata A, Kitamura H, Ohara O, Wang JY, et al. (2008) Discriminating gene expression profiles of memory B cell subpopulations. J Exp Med 205: 1807-1817.

27. Sugalski JM, Rodriguez B, Moir S, Anthony DD (2010) Peripheral blood B cell subset skewing is associated with altered cell cycling and intrinsic resistance to apoptosis and reflects a state of immune activation in chronic hepatitis C virus infection. J Immunol 185: 3019-3027.

28. Booth J, Wilson H, Jimbo S, Mutwiri G (2011) Modulation of B cell responses by Toll-like receptors. Cell Tissue Res 343: 131-140.

29. Bekeredjian-Ding I, Jego G (2009) Toll-like receptors--sentries in the B-cell response. Immunology 128: 311-323.

30. Nemazee D, Gavin A, Hoebe K, Beutler B (2006) Immunology: Toll-like receptors and antibody responses. Nature 441: E4.

31. Pasare C, Medzhitov R (2005) Control of B-cell responses by Toll-like receptors. Nature 438: 364-368.

32. Jiang W, Lederman MM, Harding CV, Rodriguez B, Mohner RJ, et al. (2007) TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. Eur J Immunol 37: 2205-2213.

33. Bernasconi NL, Traggiai E, Lanzavecchia A (2002) Maintenance of serological memory by polyclonal activation of human memory B cells. Science 298: 2199-2202.

34. Anolik JH, Looney RJ, Lund FE, Randall TD, Sanz I (2009) Insights into the heterogeneity of human B cells: diverse functions, roles in autoimmunity, and use as therapeutic targets. Immunol Res 45: 144-158.

35. Mbow M, Larkin BM, Meurs L, Wammes LJ, de Jong SE, et al. (2012) T-Helper 17 Cells Are Associated With Pathology in Human Schistosomiasis. J Infect Dis. jis654.

36. Henri S, Chevillard C, Mergani A, Paris P, Gaudart J, et al. (2002) Cytokine regulation of periportal fibrosis in humans infected with Schistosoma mansoni: IFN-gamma is associated with protection against fibrosis and TNF-alpha with aggravation of disease. J Immunol 169: 929-936.

37. Booth M, Mwatha JK, Joseph S, Jones FM, Kadzo H, et al. (2004) Periportal fibrosis in human Schistosoma mansoni infection is associated with low IL-10, low IFN-gamma, high TNF-alpha, or low RANTES, depending on age and gender. J Immunol 172: 1295-1303.

38. van den Biggelaar AH, Lopuhaa C, van Ree R, van der Zee JS, Jans J, et al. (2001) The prevalence of parasite infestation and house dust mite sensitization in Gabonese schoolchildren. Int Arch Allergy Immunol 126: 231-238.

39. van Riet E, Adegnika AA, Retra K, Vieira R, Tielens AG, et al. (2007) Cellular and humoral responses to influenza in gabonese children living in rural and semi-urban areas. J Infect Dis 196: 1671-1678.

40. Katz N, Chaves A, Pellegrino J (1972) A simple device for quantitative stool thick-smear technique in Schistosomiasis mansoni. Rev Inst Med Trop Sao Paulo 14: 397-400.

41. Polderman AM, Krepel HP, Baeta S, Blotkamp J, Gigase P (1991) Oesophagostomiasis, a common infection of man in northern Togo and Ghana. Am J Trop Med Hyg 44: 336-344.

42. Planche T, Krishna S, Kombila M, Engel K, Faucher JF, et al. (2001) Comparison of methods for the rapid laboratory assessment of children with malaria. Am J Trop Med Hyg 65: 599-602.

43. Butterworth AE, Dunne DW, Fulford AJ, Thorne KJ, Gachuhi K, et al. (1992) Human immunity to Schistosoma mansoni: observations on mechanisms, and implications for control. Immunol Invest 21: 391-407.

44. Hanten JA, Vasilakos JP, Riter CL, Neys L, Lipson KE, et al. (2008) Comparison of human B cell activation by TLR7 and TLR9 agonists. BMC Immunol 9: 39.

45. Ruprecht CR, Lanzavecchia A (2006) Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur J Immunol 36: 810-816.

46. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, et al. (2011) Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood 118: 2150-2158.

47. Grogan JL, Kremsner PG, Deelder AM, Yazdanbakhsh M (1998) Antigen-specific proliferation and interferon-gamma and interleukin-5 production are down-regulated during Schistosoma haematobium infection. J Infect Dis 177: 1433-1437.

48. Everts B, Adegnika AA, Kruize YC, Smits HH, Kremsner PG, et al. (2010) Functional impairment of human myeloid dendritic cells during Schistosoma haematobium infection. PLoS Negl Trop Dis 4: e667.

49. Nausch N, Midzi N, Mduluza T, Maizels RM, Mutapi F (2011) Regulatory and activated T cells in human Schistosoma haematobium infections. PLoS One 6: e16860.

50. Wojciechowski W, Harris DP, Sprague F, Mousseau B, Makris M, et al. (2009) Cytokine-producing effector B cells regulate type 2 immunity to H. polygyrus. Immunity 30: 421-433.

51. Menard LC, Minns LA, Darche S, Mielcarz DW, Foureau DM, et al. (2007) B cells amplify IFN-gamma production by T cells via a TNF-alpha-mediated mechanism. J Immunol 179: 4857-4866.

52. Correa-Oliveira R, Caldas IR, Gazzinelli G (2000) Natural versus drug-induced resistance in Schistosoma mansoni infection. Parasitol Today 16: 397-399.

53. Onguru D, Liang Y, Elliot J, Mwinzi P, Ganley-Leal L (2011) CD23b isoform expression in human schistosomiasis identifies a novel subset of activated B cells. Infect Immun 79: 3770-3777.

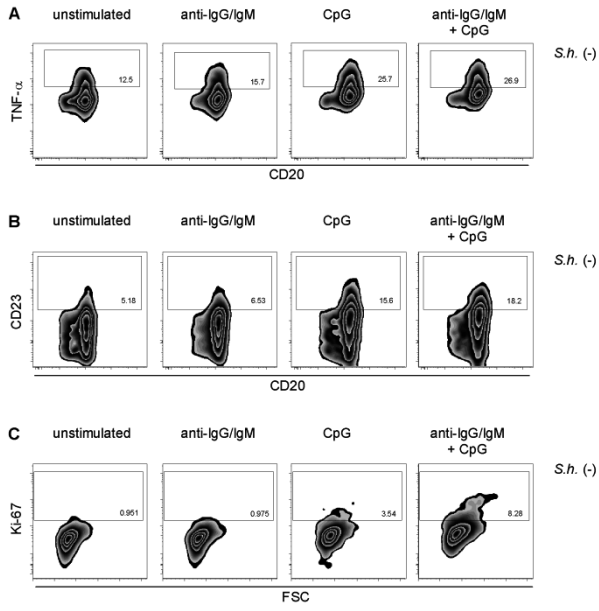
54. Griffith QK, Liang Y, Onguru DO, Mwinzi PN, Ganley-Leal LM (2011) CD23-bound IgE augments and dominates recall responses through human naive B cells. J Immunol 186: 1060-1067.

55. Bulati M, Buffa S, Candore G, Caruso C, Dunn-Walters DK, et al. (2011) B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans. Ageing Res Rev 10: 274-284.

56. Rodriguez-Bayona B, Ramos-Amaya A, Perez-Venegas JJ, Rodriguez C, Brieva JA (2010) Decreased frequency and activated phenotype of blood CD27 IgD IgM B lymphocytes is a permanent abnormality in systemic lupus erythematosus patients. Arthritis Res Ther 12: R108.

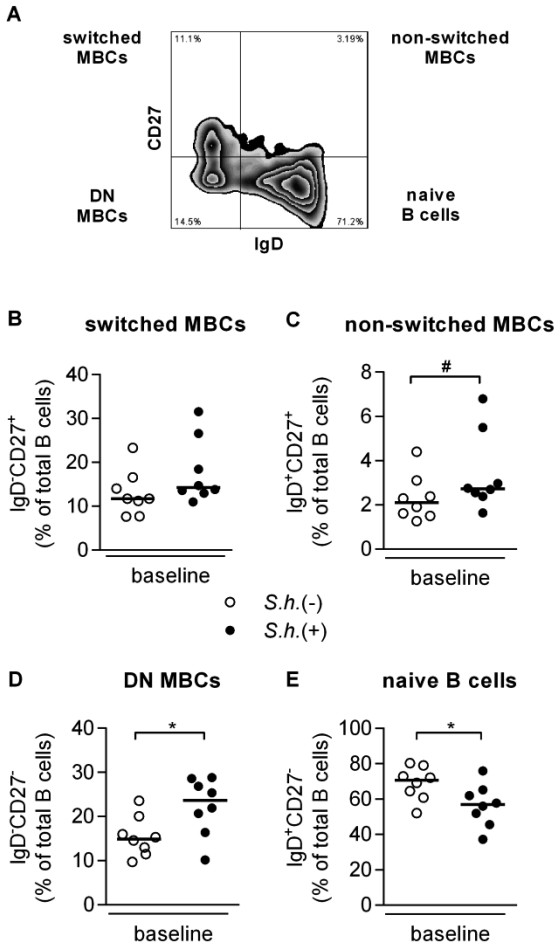
57. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, et al. (2012) B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J Exp Med 209: 1001-1010.

SUPPORTING INFORMATION LEGENDS



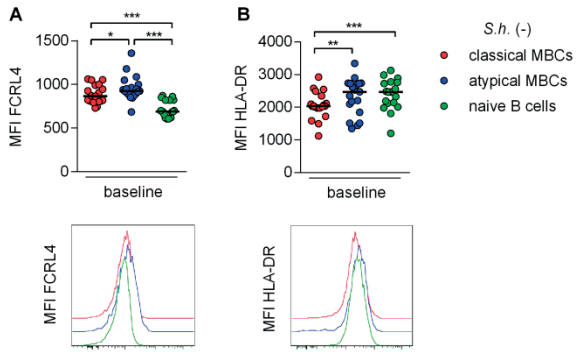
Supplementary Figure 1. ***Gating strategy for B cell inflammatory cytokine response, activation and proliferation.***

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, naive 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

Chapter 4

Associations between helminth infections, *Plasmodium falciparum* parasite carriage and antibody responses to sexual and asexual stage malaria antigens

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Abstract

Infections with helminths and *Plasmodium spp.* overlap in their geographical distribution. It has been postulated that helminth infections may influence malaria transmission by altering *P. falciparum* gametocytogenesis. This cross-sectional study assessed the effect of helminth infections on *P. falciparum* gametocyte carriage and on humoral immune responses to sexual stage antigens in Gabon. *Schistosoma haematobium* and filarial infections as well as *Plasmodium falciparum* asexual forms and gametocyte carriage were determined. The antibody responses measured were to sexual (Pfs230, Pfs48/45) and asexual *P. falciparum* antigens (AMA1, MSP1, GLURP).

A total of 287 subjects were included. The prevalence of microscopically detectable *P. falciparum* asexual parasites was higher in *S. haematobium* infected subjects in comparison to their uninfected counterparts (47% vs 26% p =0.003), but this was not different when filarial infections were considered. *P. falciparum* gametocyte carriage was similar between schistosoma or filaria infected *versus* uninfected subjects. We observed a significant decrease of Pfs48/45 IgG titre in *S. haematobium* infected subjects (p=0.037) whereas no difference was seen for Pfs230 antibody titre, nor for antibodies to AMA1, MSP1 or GLURP.

Our findings suggest an effect of *Schistosoma haematobium* on antibody responses to some *P. falciparum* gametocyte antigens that may have consequences for transmission-blocking immunity.

Introduction

In many malaria endemic regions, helminth infections are also prevalent, thereby affecting the same population [1,2]. There is some evidence suggesting an interaction between helminth and *Plasmodium spp*, however, this has not been consistent. For example the prevalence and severity of malaria as well as *P. falciparum* parasitemia density has been reported to be higher [3,4] in some but lower [5,6] in other studies comparing helminth-infected subjects with those uninfected. Similarly, at the immunological level, there are conflicting reports regarding the effect of chronic helminth infections on the immune responses to *Plasmodium spp*. [7,8]. The immunological protection against clinical malaria episodes is associated with a more pronounced Th1 response [9] and with the production of cytophilic antibodies (IgG1 and IgG3) [10,11]. However the immune phenotype of helminth-infected subjects is generally characterized by a Th2 skewed response [12,13] and is marked by the production of non cytophilic IgG (IgG4) and IgE antibodies [14]. Helminth infections have also been shown to induce a strong regulatory network that can dampen the immune response to unrelated antigens like those from *Plasmodium spp.* parasites [15,16]. One may therefore speculate that malaria specific immune responses may be impaired in subjects chronically infected with helminths. However studies that have assessed this question have yielded conflicting results indicating that larger and better designed studies are needed [17–20].

To date, studies assessing the co-infection of helminths and malaria have mainly focused on the asexual forms of *P. falciparum* (reviewed in [21]). However there are indications that helminth infections may also influence the prevalence or density of *P. falciparum* gametocytes, the parasite stage responsible for transmission of infections to mosquitoes [22]. Studies in Africa and Asia have reported an increased prevalence of *P. falciparum* gametocyte carriage in helminth infected subjects [22,23]. Interestingly using a murine model of coinfection Noland et al. showed that transmission of gametocytes from mice to mosquitoes was higher when mosquitoes were fed on helminth and malaria coinfected mice [24]. Taken together these findings might indicate a role for helminths in sustaining malaria transmission in co-endemic areas.

Towards understanding the effect of helminths on malaria immunity and transmissibility we have conducted a cross-sectional study in an area

endemic for both helminths and malaria. In this study, we have assessed the effect of *S. haematobium* and filarial parasites on the prevalence of sexual and asexual forms of *P. falciparum* parasite. Finally we determined the association between helminth and malaria co-infections on the humoral responses to sexual stage antigens Pfs230 and Pfs48/45, along with a panel of asexual stage antigens (AMA1, MSP1 and GLURP).

Methods

Study population study area and study procedure

The study participants were selected among the population of the Zilé village in the Moyen-Ogooué province (Gabon). This village is endemic for *Schistosoma haematobium*, *Loa loa* and *Mansonella perstans* as well as for various geohelminths [25–27]. Malaria is also endemic in the area with *P. falciparum* reported as dominant species [28]. Inclusion of participants in the study was not random but rather based on their willingness to participate. Participants were recruited at home during field visits of the study team. Urine and blood samples collected in EDTA tubes were taken for all the subjects to assess for schistosoma, filaria and malaria infection. Venous blood was drawn and serum was obtained for ELISA.

Parasitological diagnosis

Plasmodium spp. infection was determined by microscopic examination of thick blood smears. Asexual forms of the parasite were detected by the Lambaréné method as described elsewhere [29]. The presence of *P. falciparum* gametocytes was established using the WHO method after counting 1000 leukocytes. DNA extraction and real time PCR (qPCR) was carried on to detect sub-microscopic infection by asexual stage *P. falciparum* as previously described [30].

Schistosoma haematobium infection was determined before inclusion in the study. Schistosoma eggs were sought in 10 ml of fresh urine passed through a 12-µm pore-size filter. Absence of infection was set after the negativity of three urines samples collected after three consecutive days. A subject was classified as infected if at least one egg was detected in the urine sample.

L. loa and *M. perstans* microfilaria was detected by a modified Knott method [31]. Microfilaria count was determined by microscopy and difference between species was established based on the presence of the sheath of *Loa loa*.

ELISA

Pfs48/45-10C was obtained from the chimeric R0-10C vaccine protein [32]. R0 was cleaved from Pfs48/45-10C and successful removal of GLURP-R0 was confirmed by testing plasma samples from GLURP-vaccinated volunteers [33]. Pfs230-230CMB was obtained from Fraunhofer USA Center for Molecular Biotechnology. Apical membrane antigen (AMA-1 3D7, Biomedical Primate Research Centre, Rijswijk, the Netherlands), merozoite surface protein 119 (MSP-119 Wellcome allele, provided by Patrick Corran, London School of Hygiene & Tropical Medicine with permission of Tony Holder) and R2 region of GLURP, provided by Michael Theisen, Statens Serum Institut, Copenhagen.

AMA-1, MSP-1 and GLURP ELISAs were performed as described previously [34]. Pfs48/45 and Pfs230 antibodies were quantified as follows; 96 well Maxisorp NUNC plates (Nalge Nunc International Corp., Naperville, IL, USA) were coated overnight at 4°C with 100µl per well of 0.1µg/ml of antigen diluted in PBS. Plates were blocked for 30 minutes with 150µl of 5% non-fat skimmed milk (Marvel, Premier International Foods Ltd., Spalding, UK) in PBS. Following this, plates were washed 3 times with PBS, and 100µl of test serum was diluted to 1/500 in PBS (with 1% milk and 0.05% Tween 20), and incubated on the plates for 4 hours at room temperature. Plates were then washed 3 times as before and incubated with 100µl per well of human-IgG-HRP (Pierce Biotechnology Inc., Rockford, IL, USA) diluted to 1/40,000 in PBS with 0.05% Tween 20, for 2 hours at room temperature. Next plates were washed 4 times, then 100µl of tetramethylbenzidine substrate (TMB) solution was added per well and incubated for 20 minutes. Reactions were stopped using 50µl per well of 0.2M sulphuric acid and optical densities were measured at 450nm (Bio-Rad iMark Microplate Reader, Hertfordshire, UK). For all assays, averaged sample ODs were normalized (using the midpoint dilution as reference), against a titration curve fitted to the positive control sample by least squares minimisation using a three variable sigmoid model [34,35]. The mixture model was used to distinguish positive and negative samples by fitting test sample ODs to

two Gaussian distributions using maximum likelihood methods in STATA (Version 11, Statacorp, Texas, USA). The mean OD of the seronegative (the test samples with low ODs) population plus 3 standard deviations was used as the cut off value. Antibody densities were expressed as % of the reference value (i.e. the normalised value).

Statistical analysis

The statistical analysis was conducted using STATA (Version 11, Statacorp, Texas, USA) and R (Version 3.0.1, R core team, Vienna, Austria). Chi square test was used for comparison of proportion. Continuous data that were not normally distributed were transformed either using a log10 transformation or a Box-Cox transformation when appropriate. Comparison of mean was carried out using the student t test or the ANOVA test for normally distributed data or the Man Whitney and the Kruskal Wallis test otherwise. Multivariable linear regression analysis was performed to assess the relationship between infectious status and the antibody response specific to *P. falciparum* gametocyte antigens. Significance level was set for a p value < 0.05.

Ethics

The study was approved by the "Comité d'éthique Régional de Lambaréné" (CERIL). Informed consent was obtained from each participant and in case they had less than 18 years old from their parents or legal guardians. Appropriate treatment was given to children found with *P. falciparum* or *S. haematobium* infection as per the local guidelines.

Results

A total of 287 participants were included in this study. Among them, 229 (81%) had either *Schistosoma haematobium* or one of the filarial infections, *M. perstans* or *L. loa*, while 197 (75%) carried *P. falciparum* parasite as determined by PCR or 120 (42%) as determined by microscopy. None of the participants carrying *P. falciparum* were symptomatic for malaria. Co-infection with plasmodium and with one or more helminth species was found in 155 (55%) subjects (Table 1).

Table 1: Characteristics of the study population

Characteristics		% (n/N)
Age (in years): Median (IQR)		11 (8-15)
Sex*: M/F		153/134
Hemoglobin level (in g/dl): Median (IQR)		11.3(10.5-12.2)
Subjects with *P. falciparum* asexual stage infection (diagnosed by microscopy)		42 (120/285)
Subjects with *P. falciparum* asexual stage infection (diagnosed by PCR)		75 (197/262)
P. falciparum gametocyte carriers		19 (52/275)
Subjects with *S. haematobium* infection		75 (214/284)
Subjects with Filaria infection**		28 (81/287)
Helminth infection status	Subjects with no helminth infection	20 (55/284)
	Subjects infected with one helminth specie	57 (163/284)
	Subjects infected with more than one helminth species	23 (66/284)
Subjects with *P. falciparum* and helminth coinfection		55 (155/284)

*M= male, F=Female, ** *loa loa* and *Mansonella perstans*

The prevalence of microscopic *P. falciparum* asexual parasites was higher in *S. haematobium* infected subjects in comparison to those free of *S. haematobium* infection (47% vs 26% p = 0.003, Table 2) while *P. falciparum* gametocyte carriage was similar between helminth infected and uninfected subjects (Table 2). Also when malaria parasites were detected by PCR, we observed a higher malaria prevalence in *S. haematobium* infected individuals, albeit not statistically significant (p=0.07; Table 2). We did not detect any differences when comparing those with and without filarial infections (table 2). The absence of association between filaria and carriage of sexual and asexual forms of *P. falciparum* remained even after correction for age in a multivariable analysis (data not shown). In this study, we measured the total IgG response of the participants to 3 asexual (MSP1, AMA1 and GLURP) and two sexual (Pfs48/45 and Pfs230) *P. falciparum* antigens. We did not observe a statistically significant effect of age or gender on the level of the different antibodies (data not shown). Asexual *P. falciparum* parasites, as determined by microscopy, did not influence the level of the five antibodies measured.

Table 2: Characteristics of the study population stratified by helminth infections

	S. haematobium infection status		*p*	Filaria infection status*		*p*	
	Uninfected subjects	Infected subjects		Uninfected subjects	Infected subjects		
Age in years: Median (IQR)	10(6)	11(6.7)	0.9	10(5)	14(8)	<0.001	
Sex (M/F)**	38/32	112/102	0.2	113/93	40/41	0.4	
Haemoglobin level (g/dl): Mean (SD)	11.6 (1.3)	11.2 (1.45)	0.06	11.3 (1.3)	11.5 (1.6)	0.3	
Subjects with *P. falciparum* asexual stage infection (diagnosed by microscopy): n (%)	18/70 (26%)	100/214 (47%)	0.003	86/206 (42%)	34/81 (43%)	1	
Subjects with *P. falciparum* asexual stage infection (diagnosed by PCR): n (%)	45(66.2%)	146 (78%)	0.07	136 (74%)	58 (78%)	0.55	
P. falciparum gametocyte carriers: n (%)	14(20.1%)	36 (18%)	0.8	41 (21%)	11 (14%)	0.2	

**loa loa and Mansonella perstans*, **M= male, F=Female

However, when *P. falciparum* asexual form was determined by PCR, higher antibody concentrations were observed in infected subjects compared to uninfected for Pfs230 (65.7 [95%CI: 59.3-72.5] vs 51.1 [95%CI: 42.7-60], p= 0.01) and AMA1 (1430.8 [95%CI: 1064.3-1904.9] vs 689.9 [95%CI: 391.7-1147.3] p= 0.01) but not for other antibodies, as shown in Table 3.Moreover, we found that+ carriage of *P. falciparum* gametocytes was associated with a trend toward increased antibodies to Pfs48/45 in gametocyte-positive (55.1 [95% CI 46.2-66.2] compared to gametocyte-negative individuals (44.7 [95%CI 40.4-49.3]; p = 0.056). The same trend was observed for Pfs230 (72[95%CI 60.5-84.4] in gametocyte-positive and 59.8 [95%CI 54.1-65.6] in gametocyte-negative individuals, p = 0.068) as shown in Table 3.

Regarding helminth infections, we observed a significant decrease of Pfs48/45 IgG titres in *S. haematobium* infected subjects compared to those uninfected (44.2 [95%CI 39.7-49.2] vs 53.2 [95%CI 46.3-60.7], p = 0.037) whereas no difference was seen for Pfs230 antibody titres, nor for the other antibodies as shown in Table 3. In contrast to *S. haematobium*, filarial infection was not associated with a significant effect on the concentration of antibodies to sexual or asexual *P. falciparum* stage antigens.

Table 3: Effect of malaria and helminth infection as well as malaria exposure on the level of total IgG specific to Pfs48/45, Pfs230, GLURP, MSP1 and AMA1

		Pfs48/45 Mean (±SD)	*p*	Pfs230 Mean (±SD)	*p*	GLURP Mean (±SD)	*p*	MSP1 Mean (±SD)	*p*	AMA1 Mean (±SD)	*p*
P. falciparum asexual stage infection status (diagnosed by microscopy)	Uninfected	46 (6.3)	0.8	59.5 (14.3)	0.3	55.9 (5.9)	0.25	54.8 (6.7)	0.09	1064.7 (12.5)	0.14
	Infected	47(6.5)		65.1 (15.6)		71.7 (5.9)		36.9 (7.2)		1533.8 (17.9)	
P. falciparum asexual stage infection status (diagnosed by PCR):	Uninfected	40.2 (5.5)	0.068	51.1 (11.5)	0.01	48.6 (5.7)	0.16	53(7)	0.38	689.9 (10)	0.01
	Infected	48.7 (6.6)		65.7 (16.2)		69.2 (6)		41.3(7)		1430.8 (17)	
P. falciparum gametocyte carriage status	Non carriers	44.7 (6.4)	0.056	59.8 (15.1)	0.068	58.2 (6)	0.36	47.6 (6.8)	0.64	1226 (16.4)	0.6
	carriers	55.1 (5.7)		72 (13)		74 (5)		41.2 (7.7)		1439 (10.5)	
S. haematobium infection status	Uninfected	53.2 (4.3)	0.037	63.3 (12.3)	0.7	69 (4.7)	0.53	55.3 (5.2)	0.33	1372.2 (11.4)	0.6
	Infected	44.2 (7)		61.3 (15.7)		60 (6.4)		43.7 (7.3)		1205.8 (16.2)	
Filaria infection status*	Uninfected	46.5 (6.4)	0.9	62.2 (15.4)	0.8	61 (5.4)	0.79	47.8 (7)	0.68	1169.1 (15.9)	0.4
	Infected	46.2 (6.2)		60.9 (13.5)		65 (7.5)		43.1(7)		1455.3(12.4)	

**Loa loa and Mansonela perstans*

To further assess how schistosome infection affects the humoral responses to *P. falciparum* sexual and asexual stage antigens, we performed a multivariable analysis on the titre of antibodies against the five antigens. In this analysis asexual *P. falciparum* infection diagnosed by PCR, *P. falciparum* gametocyte carriage, hemoglobin concentration and participant age was used as predictor variables. These predictor variables were selected based on their reported effect on gametocyte carriage and on antibodies specific to *P. falciparum* asexual or sexual stage antigens.

Table 4 : Multivariable linear regression analysis assessing the effect of *S. haematobium* infection, *P. falciparum* infection as detected by PCR, haemoglobin level as well as age on antibodies to *P. falciparum* sexual and asexual stage antigens. Three models were considered for this analysis. In the first model all subjects were included regardless of whether or not they were infected with *S. haematobium*. Subsequent analysis focus on *S. haematobium* uninfected (model 2) or infected subjects (model 3).

Antibody	Covariates	***S. haematobium* infection status**					
		All subjects		*S. haematobium* uninfected subjects		*S. haematobium* infected subjects	
		β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
Pfs48/45	*S. haematobium* infection	0.68 (0.48-0.97)	0.0352	-	-	-	-
	Infection with asexual forms of *P. falciparum*	1.14 (0.78-1.67)	0.487	1.85 (1.25-2.74)	0.003	0.93 (0.56-1.55)	0.773
	Carriage of sexual forms of *P. falciparum*	1.41 (0.93-2.15)	0.106	1.16 (0.73-1.85)	0.529	1.5 (0.87-2.6)	0.142
	Hemoglobin level	1 (0.89-1.13)	0.95	1.02 (0.89-1.18)	0.748	1.01 (0.87-1.17)	0.946
	Age	1.01 (0.99-1.03)	0.125	1.02 (1-1.03)	0.03	1.01 (0.99-1.04)	0.316
Pfs230	*S. haematobium* infection	0.79 (0.54-1.17)	0.237	-	-	-	-
	Infection with asexual forms of *P. falciparum*	1.19 (0.78-1.81)	0.412	1.91 (1.1-3.34)	0.023	0.93 (0.54-1.61)	0.792
	Carriage of sexual forms of *P. falciparum*	1.30 (0.82-2.07)	0.264	1.37 (0.71-2.66)	0.348	1.26 (0.7-2.27)	0.431
	Hemoglobin level	0.98 (0.86-1.12)	0.785	0.97 (0.79-1.19)	0.757	0.98 (0.84-1.16)	0.86
	Age	1.01 (0.99-1.03)	0.261	1.01 (0.99-1.03)	0.345	1.01 (0.99-1.04)	0.293

Table 4 ***(contd)*** **:** Multivariable linear regression analysis assessing the effect of *S. haematobium* infection, *P. falciparum* infection as detected by PCR, haemoglobin level as well as age on antibodies to *P. falciparum* sexual and asexual stage antigens. Three models were considered for this analysis. In the first model all subjects were included regardless of whether or not they were infected with *S. haematobium*. Subsequent analysis focus on *S. haematobium* uninfected (model 2) or infected subjects (model 3).

Antibody	Covariates	***S. haematobium* infection status**					
		All subjects		*S. haematobium* uninfected subjects		*S. haematobium* infected subjects	
		β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
AMA1	*S. haematobium* infection	0.64 (0.3-1.35)	0.242	-	-	-	-
	Infection with asexual forms of *P. falciparum*	2.02 (0.9-4.53)	0.085	3.74 (1.08-12.93)	0.038	1.56 (0.55-4.38)	0.4
	Carriage of sexual forms of *P. falciparum*	1.25 (0.51-3.05)	0.629	1.41 (0.32-6.17)	0.645	1.16 (0.38-3.52)	0.786
	Hemoglobin level	1.03 (0.8-1.32)	0.846	1.24 (0.79-1.95)	0.344	0.97 (0.72-1.32)	0.86
	Age	1.03 (0.99-1.07)	0.085	1.03 (0.98-1.08)	0.258	1.04 (0.99-1.09)	0.144
MSP1	*S. haematobium* infection	0.76 (0.43-1.36)	0.357	-	-	-	-
	Infection with asexual forms of *P. falciparum*	0.69 (0.37-1.29)	0.248	0.79 (0.3-2.1)	0.633	0.66 (0.3-1.47)	0.312
	Carriage of sexual forms of *P. falciparum*	0.92 (0.46-1.85)	0.822	0.61 (0.19-1.96)	0.4	1.06 (0.45-2.48)	0.9
	Hemoglobin level	0.91 (0.75-0.11)	0.363	0.99 (0.69-1.41)	0.95	0.89 (0.71-1.13)	0.341
	Age	1.01 (0.98-1.04)	0.492	0.998 (0.96-1.04)	0.928	1.02 (0.98-1.06)	0.4
GLURP	*S. haematobium* infection	0.81 (0.45-1.32)	0.399	-	-	-	-
	Infection with asexual forms of *P. falciparum*	1.52 (0.9-2.56)	0.116	2.17 (0.9-5.21)	0.082	1.34 (0.69-2.56)	0.386
	Carriage of sexual forms of *P. falciparum*	1.36 (0.76-2.42)	0.303	1.37 (0.48-3.89)	0.551	1.33 (0.66-2.68)	0.427
	Hemoglobin level	0.99 (0.84-1.16)	0.878	1.19 (0.87-1.64)	0.276	0.94 (0.77-1.14)	0.522
	Age	1.03 (1-1.05)	0.027	1.02 (0.99-1.06)	0.186	1.03 (0.99-1.06)	0.068

We fitted the model for the total population as well as for *S. haematobium* uninfected and infected individuals, separately. In the total population, we observed no apparent association between Pfs230, AMA1, GLURP or MSP1 antibody responses and *S. haematobium* infection (Table 4) but we did find a significant decrease of Pfs48/45 antibody titre in *Schistosoma* infected subjects compared to their uninfected counterparts (β= 0.68, 95%CI: 0.48-0.97 , p=0.035, Table 4). When restricting the analysis to subjects not infected with *S. haematobium,* we observed that *P. falciparum* infection as determined by PCR was associated with a significant increase of Pfs48/45 (β=1.84, 95%CI: 1.2-2.7, p=0.003, Table 4) and Pfs230 specific antibodies (β=1.9, 95%CI: 1.2-3.3, p=0.02) as shown in Table 4. This association was not seen when the model was applied to those infected with *S. haematobium* (Table 4).

Discussion

To our knowledge this is the first study to assess the effect of helminth infections on both the carriage and the humoral immune response to *P. falciparum* sexual stage antigens. Our primary interest was to determine if gametocyte production, and antibody responses to gametocyte antigens, was higher in malaria-schistosome co-infected subjects. What we observed is that whilst gametocyte carriage as determined by microscopy did not differ between individuals infected with malaria alone and with malaria and *S. haematobium*, antibody levels to Pfs48/45 sexual stage antigen but not to Pfs230 were lower in co-infected individuals. Our initial hypothesis, based on the literature, was that a higher prevalence of *P. falciparum* gametocytes and markers of gametocyte exposure would be found in *S. haematobium* co-infected individuals [22,23]. Contrary to this hypothesis, we observed no apparent effect of *S. haematobium* co-infection on gametocyte carriage. This could be explained by the fact that we used microscopy to detect gametocytes, which is less sensitive than molecular methods [36]. It is possible that molecular detection methods for gametocytes or functional assays (e.g. standard membrane feeding) could have given a different picture in line with what has been described in two studies in the literature showing that helminth infections increase gametocyte carriage [22,23]. A more sensitive marker of gametocyte exposure than

microscopy might be antibodies to two gametocyte antigens, Pfs230 and Pfs48/45 [37,38]. Antibody responses to these molecules indeed appeared to be elevated in patent gametocyte carriers, suggesting that they may serve as specific markers of gametocyte exposure. Interestingly, in the current study, lower levels of antibodies to Pfs48/45 but not to Pfs230 were seen in subjects with schistosome infection. This suggests either a spurious finding, a selective suppressory effect of *Schistosoma* co-infection on antibody responses against Pfs48/45 or lower immunogenicity of Pfs48/45 and amenable to modulation. In line with our finding, a study in Papua New Guinea with high transmission of malaria showed that if sera recognized the gametocyte surface antigens, the response was dominated by antibodies to Pfs230 with fewer people showing a response to pfs48/45 [39]. However helminth infections which are highly prevalent in the area were not considered and therefore it is not possible to delineate whether the low response to Pfs48/45 is due to the presence of helminth infections.

In our study we noted a higher percentage of schistosoma infected participants was infected with *P. falciparum*, this was statistically significant when parasites were detected by microscopy but fell short of significance when PCR was used to detect malaria parasites. One way to interpret this is that schistosome infections are associated with higher burden of malaria parasites. The higher carriage of *P. falciparum* suggests a possible interaction between helminths and *P. falciparum*, which is unlikely to be explained by structural features of the houses, an important factor in malaria transmission, as houses in our study area were very similar to each other. Other factors such as nutritional status or proximity to water bodies could explain such an interaction but we did not collect data on these parameters.

We did not observe an effect of schistosomiasis on the antibody titres to *P. falciparum* asexual stage antigens, AMA1, GLURP or MSP1. This is in line with the results from Lyke *et al.* [7] in Mali but it contrasts to the observations of Diallo *et al.* in Senegal who reported a significant increase of the humoral response to plasmodium antigens in *Schistosoma* infected subjects [8]. A plausible explanation of these differences might lie in the characteristics of the populations studied as well as in the epidemiological feature of malaria and schistosomiasis in

the different study areas. For instance in the study in Mali all participants came from an area where, like in our study area, both malaria and schistosomiasis show intense seasonal transmission [7]. In contrast the study in Senegal was conducted in an area of low malaria transmission and helminth free subjects were recruited from a village where *S. haematobium* had never been reported before and was absent at the time of the study [8]. Because exposure to these parasites is expected to be very different in the two studies, this could affect the immune response profiles measured. Finally it is also important to emphasize that Diallo et al. measured the level of the different IgG subclasses whereas Lyke et al. and ourselves studied total IgG. Future studies should examine the effect of helminths on IgG subclasses since protection to malaria is linked with an increase of cytophilic antibodies of IgG1 and IgG3 subclasses. Why filarial infections did not affect the antibody responses to malarial antigens is not clear. Species dependent effect of helminth on malariometric indices and on malaria specific immune response have already been reported [40,41]. It is possible that schistosomiasis has a more pronounced effect on the host immune response and/or metabolism than filarial infections. In this study we observed for example that *Schistosoma* but not filaria infected subjects were more likely to be anemic. Future studies will need to expand and further assess the effect of filaria or other helminths species on malaria transmission

The present study should be regarded as hypothesis generating and calls for additional work to assess the association between helminths and malaria transmission. Future studies should ideally have a longitudinal design and include the collection of material for sensitive gametocyte detection by RNA-based methods [42]. Future studies should also determine the functional importance of the observed decrease of Pfs48/45 specific IgG in schistosoma infected subjects using mosquito feeding assays.

In summary this study suggests a selective effect of *S. haematobium* on the humoral response to an important *P. falciparum* sexual stage antigen. It indicates an association between helminths and malaria transmission and may form a starting point for more detailed studies on the consequences of dual infections for disease transmission.

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References

1.Mazigo, H. D. *et al.* Co-infections with Plasmodium falciparum, Schistosoma mansoni and intestinal helminths among schoolchildren in endemic areas of northwestern Tanzania. *Parasit Vectors* **3,** 44 (2010).

2.Brooker, S. *et al.* Epidemiology of plasmodium-helminth co-infection in Africa: populations at risk, potential impact on anemia, and prospects for combining control. *Am. J. Trop. Med. Hyg.* **77,** 88–98 (2007).

3.Mulu, A. *et al.* Epidemiological and clinical correlates of malaria-helminth co-infections in Southern Ethiopia. *Malar. J.* **12,** 227 (2013).

4.Sangweme, D. T. *et al.* Impact of schistosome infection on Plasmodium falciparum Malariometric indices and immune correlates in school age children in Burma Valley, Zimbabwe. *PLoS Negl Trop Dis* **4,** e882 (2010).

5.Lyke, K. E. *et al.* Association of Schistosoma haematobium infection with protection against acute Plasmodium falciparum malaria in Malian children. *Am. J. Trop. Med. Hyg.* **73,** 1124–1130 (2005).

6.Lemaitre, M. *et al.* Coinfection with Plasmodium falciparum and Schistosoma haematobium: Additional Evidence of the Protective Effect of Schistosomiasis on Malaria in Senegalese Children. *Am J Trop Med Hyg* **90,** 329–334 (2014).

7.Lyke, K. E. *et al.* Antigen-specific B memory cell responses to Plasmodium falciparum malaria antigens and Schistosoma haematobium antigens in co-infected Malian children. *PLoS ONE* **7,** e37868 (2012).

8.Diallo, T. O. *et al.* Schistosomiasis coinfection in children influences acquired immune response against Plasmodium falciparum malaria antigens. *PLoS ONE* **5,** e12764 (2010).

9.Torre, D. *et al.* Role of Th1 and Th2 cytokines in immune response to uncomplicated Plasmodium falciparum malaria. *Clin. Diagn. Lab. Immunol.* **9,** 348–351 (2002).

10.Bouharoun-Tayoun, H. & Druilhe, P. Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infect. Immun.* **60,** 1473–1481 (1992).

11.Courtin, D. *et al.* The Quantity and Quality of African Children's IgG Responses to Merozoite Surface Antigens Reflect Protection against Plasmodium falciparum Malaria. *PLoS One* **4,** (2009).

12.Winkler, S. *et al.* Increased frequency of Th2-type cytokine-producing T cells in microfilaremic loiasis. *Am. J. Trop. Med. Hyg.* **60,** 680–686 (1999).

13.Maizels, R. M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat. Rev. Immunol.* **3,** 733–744 (2003).

14.Hagan, P., Blumenthal, U. J., Dunn, D., Simpson, A. J. & Wilkins, H. A. Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium. *Nature* **349,** 243–245 (1991).

15.Metenou, S. *et al.* Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. *J. Immunol.* **186,** 4725–4733 (2011).

16.Wammes, L. J. *et al.* Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. *Eur. J. Immunol.* **40,** 437–442 (2010).

17.Dolo, H. *et al.* Filariasis attenuates anemia and proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults. *PLoS Negl Trop Dis* **6,** e1890 (2012).

18.Noone, C. *et al.* Plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with Plasmodium falciparum. *Malar. J.* **12,** 5 (2013).

19.Lyke, K. E. *et al.* Effects of concomitant Schistosoma haematobium infection on the serum cytokine levels elicited by acute Plasmodium falciparum malaria infection in Malian children. *Infect. Immun.* **74,** 5718–5724 (2006).

20.Metenou, S. *et al.* Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. *J. Immunol.* **186,** 4725–4733 (2011).

21.Nacher, M. Helminth-infected patients with malaria: a low profile transmission hub? *Malar. J.* **11,** 376 (2012).

22.Nacher, M. *et al.* Association of helminth infections with increased gametocyte carriage during mild falciparum malaria in Thailand. *Am. J. Trop. Med. Hyg.* **65,** 644–647 (2001).

23.Sangweme, D. T. *et al.* Impact of schistosome infection on Plasmodium falciparum Malariometric indices and immune correlates in school age children in Burma Valley, Zimbabwe. *PLoS Negl Trop Dis* **4,** e882 (2010).

24.Noland, G. S., Graczyk, T. K., Fried, B. & Kumar, N. Enhanced malaria parasite transmission from helminth co-infected mice. *Am. J. Trop. Med. Hyg.* **76,** 1052–1056 (2007).

25.Akue, J. P. *et al.* Epidemiology of concomitant infection due to Loa loa and Mansonella perstans in Gabon. *PLoS Negl Trop Dis* **5,** e1329 (2011).

26.Adegnika, A. A. *et al.* Epidemiology of parasitic co-infections during pregnancy in Lambaréné, Gabon. *Tropical Medicine & International Health* **15,** 1204–1209 (2010).

27.Ngoa, U. A. *et al.* Assessment of the effect of Schistosoma haematobium co infection on malaria parasites and immune responses in rural populations in Gabon: study protocol. *SpringerPlus* **3,** 388 (2014).

28.Wildling, E. *et al.* Malaria epidemiology in the province of Moyen Ogoov, Gabon. *Trop. Med. Parasitol.* **46,** 77–82 (1995).

29.Planche, T. *et al.* Comparison of Methods for the Rapid Laboratory Assessment of Children with Malaria. *Am J Trop Med Hyg* **65,** 599–602 (2001).

30.Adegnika, A. A. *et al.* Microscopic and Sub-Microscopic Plasmodium Falciparum Infection, but Not Inflammation Caused by Infection, Is Associated with Low Birth Weight. *Am J Trop Med Hyg* **75,** 798–803 (2006).

31. Goldsmid, J. M. Studies on the laboratory diagnosis of human filariasis: preliminary communication. *J. Clin. Pathol.* **23,** 632–635 (1970).

32.Theisen, M. *et al.* A multi-stage malaria vaccine candidate targeting both transmission and asexual parasite life-cycle stages. *Vaccine* **32,** 2623–2630 (2014).

33.Hermsen, C. C. *et al.* Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of Plasmodium falciparum in a phase 1 malaria vaccine trial. *Vaccine* **25,** 2930–2940 (2007).

34.Drakeley, C. J. *et al.* Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 5108–5113 (2005).

35.Proietti, C. *et al.* Influence of infection on malaria-specific antibody dynamics in a cohort exposed to intense malaria transmission in northern Uganda. *Parasite Immunol.* **35,** 164–173 (2013).

36.Bousema, T. & Drakeley, C. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. *Clin. Microbiol. Rev.* **24,** 377–410 (2011).

37.Bousema, J. T. *et al.* A longitudinal study of immune responses to Plasmodium falciparum sexual stage antigens in Tanzanian adults. *Parasite Immunol.* **29,** 309–317 (2007).

38.Bousema, T. *et al.* The dynamics of naturally acquired immune responses to Plasmodium falciparum sexual stage antigens Pfs230 & Pfs48/45 in a low endemic area in Tanzania. *PLoS ONE* **5,** e14114 (2010).

39.Carter, R., Graves, P. M., Quakyi, I. A. & Good, M. F. Restricted or absent immune responses in human populations to Plasmodium falciparum gamete antigens that are targets of malaria transmission-blocking antibodies. *J. Exp. Med.* **169,** 135–147 (1989).

40.Nacher, M. Interactions between worms and malaria: good worms or bad worms? *Malar. J.* **10,** 259 (2011).

41.Supali, T. *et al.* Polyparasitism and its impact on the immune system. *Int. J. Parasitol.* **40,** 1171–1176 (2010).

42.Wampfler, R. *et al.* Strategies for detection of Plasmodium species gametocytes. *PLoS ONE* **8,** e76316 (2013).

Chapter 5

$CD4^+CD25^{hi}FOXP3^+$ cells in cord blood of neonates born from filaria infected mother are negatively associated with $CD4^+Tbet^+$ and $CD4^+ROR\gamma t^+$ T cells

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Abstract

Background

Children who have been exposed *in utero* to maternal filarial infection are immunologically less responsive to filarial antigens, have less pathology, and are more susceptible to acquire infection than offspring of uninfected mothers. Moreover children from filaria infected mothers have been shown to be less responsive to vaccination as a consequence of an impairment of their immune response. However, it is not well known how *in utero* exposure to parasite antigens affects cellular immune responses.

Methodology

Here, 30 pregnant women were examined for the presence of microfilaria of *Loa loa* and *Mansonella perstans* in peripheral blood. At delivery, cord blood mononuclear cells (CBMC) were obtained and the $CD4^+$T cells were phenotyped by expression of the transcription factors Tbet, RORγt, and FOXP3.

Results

No significant difference was observed between newborns from infected versus uninfected mothers in the frequencies of total $CD4^+$T cells and $CD4^+$T cells subsets including $CD4^+Tbet^+$, $CD4^+ROR\gamma t^+$ T and $CD4^+CD25^{hi}FOXP3^+$ T cells. However, there was a negative association between $CD4^+CD25^{hi}FOXP3^+$T cells and $CD4^+Tbet^+$ as well as $CD4^+ROR\gamma t^+$ T cells in the infected group only (B= -0.242, P=0.002; B= -0.178, P=0.013 respectively).

Conclusion

Our results suggest that filarial infection during pregnancy leads to an expansion of functionally active regulatory T cells that keep Th1 and Th17 in check.

Introduction

Parasitic infections are highly prevalent in the tropics and when present during pregnancy can affect the immune system of an unborn child directly, through transfer of parasites or antigens across the placenta (1). The early priming of the fetal immune system by parasitic antigens, has been reported to lead to a relative impairment of the innate and adaptive immune response in the neonate and later in infancy (2–8). As a consequence neonates born from parasite infected mothers are more susceptible to infection (3) and have a weaker response to vaccines administered during the first years of their life (9). This is well illustrated in a report by Malhotra and colleagues who observed that children exposed to malaria *in utero* acquire a tolerant phenotype to *Plasmodium falciparum* blood stages antigens and have an increased susceptibility to malaria infection during childhood in comparison to their malaria unexposed relatives (3). With respect to response to vaccines, a study comparing response to BCG vaccination between children from Malawi and the UK showed an inferior response to BCG in Malawian children suggesting that pre natal exposure to environmental factors such as microorganism and parasites might in part account for the difference in the Malawian and UK infants (9). Among the infections that are highly prevalent in rural areas of the world are parasitic helminthes, which are thought to exert strong immune modulatory effects (10).

In sub-Saharan Africa a high prevalence of filarial infections, such as *Wuchereria bancrofti*, *Onchocerca Volvulus*, *Loa loa* and *Mansonella perstans* is often observed in pregnant women (11). Transplacental transfer of *Wuchereria bancrofti* (12,13) or its antigens (1) from the mother to the fetus has been reported. In the case of filariasis, a number of studies have indicated that in utero exposure to maternal filarial infections can have consequences for the child after birth. Epidemiological studies have shown children from filaria infected mothers to be more susceptible to filarial infection (5,14) and to have a higher risk of mother-to-child transmission of human immunodeficiency virus (HIV) (15) in comparison with children born to uninfected mothers. How filarial parasites alter the immune system of the fetus during pregnancy has not been studied extensively. Helminth infections in general, and filariasis in particular, are

associated with the induction of a regulatory network that dampen strong immunological reactivities (10). The role of this regulatory network has also been suggested during an *in vitro* study where the cellular responsiveness and the cytokine production of cord blood mononuclear cells (CBMCs) of newborns from filaria infected mothers were assessed (16). These CBMCs were hyporesponsive to *Onchocerca volvulus* derived antigens, along with high production of the suppressive cytokine, IL-10 (16). Studies on peripheral blood mononuclear cells (PBMCs) from adults have emphasized the association between filarial infection and regulatory T cells. For example *Babu et al.* reported that stimulation of PBMCs with live infective-stage larvae of *Brugia malayi* resulted in a more pronounced activation of the regulatory network in filaria infected subjects resulting in alterations in Th1 and Th2 responses (17). Similarly, Wammes *et al.* observed lower responsiveness of T and B cells upon stimulation with *B. malayi* antigens, as well as lower secretion of Th1- and Th2-type cytokines in *B. malayi* infected patients presenting with lymphatic filariasis compared to their uninfected counterparts (18). Interestingly the hypo-responsiveness was reversed following depletion of $CD4^{+}CD25^{hi}FOXP3^{+}$ regulatory T cells suggesting their role in dampening T cell responses (18).

$CD4^{+}CD25^{hi}FOXP3^{+}$ regulatory T cells (also known as natural T regulatory cells (nTregs)) together with adaptive T regulatory cells appear to be associated with human helminths infection (19). The nTregs develop in the thymus at an early stage of the human fetal development from $CD4^{+}CD25^{hi}$ thymocytes that can recognize self-antigens (20). Adaptive regulatory T cells, which are thought to develop in the periphery in response to exogenous antigens, can also regulate effector T cells. FOXP3 has been described as the principal transcription factor of nTregs (21) required both for the development of nTreg and the maintenance of its suppressive function (22). As for nTreg cells, the adaptive regulatory T cells can express FOXP3. Other T helper cells can also be characterized by transcription factors. This is the case for Th1- (expressing T-bet) and Th17- cells (expressing RORγt). A tight correlation between the level of transcriptional factors and cytokines secreted by terminally polarized T cells has been described, suggesting the use of transcription factors as a marker of Th cells polarization (23,24). However utilization of transcription factors

in this sense is relatively recent, and little data is available regarding their expression as well as their profiles in newborns from helminth-infected mothers.

We asked the question whether maternal filarial infection can alter the early balance between the $CD4^+$T cell subsets that are known to be involved in immune responses to malaria parasites, namely the Th1 (25,26) and Th17 (27). Therefore, we analyzed in Gabon the expression of Tbet, RORγt and FOXP3 in CBMCs from neonates born to mothers infected with *Loa loa* and/or *Mansonella perstans*, comparing them to profiles seen in neonates from uninfected mothers.

Method

This study was carried out between May and August 2011 in Fougamou, a semi-rural town located in the center of Gabon, a sub-Saharan African country. This area is known to be endemic for blood-borne filaria (*Loa loa* and *Mansonella perstans)* as well as malaria (28–30). Study participants were pregnant women. For our study, inclusion of participants was based on filaria infection. Therefore filaria infected women were asked to join the study, and for each infected woman an uninfected counterpart was included as well.

Infection status of the mother was determined during pregnancy for *Schistosoma haematobium* and microfilaria infection. On the other hand *P. falciparum* infection of the mother was assessed throughout the pregnancy, as well as at the time of delivery. Additionally the presence of Plasmodium falciparum was assessed in the cord blood and the placenta. Filarial infection (*Loa loa* and *Mansonella perstans*) was diagnosed two months before the expected date of delivery by the Leucoconcentration method and parasite count was obtained by microscopy (31). None of the mother was treated before delivery since the drugs to treat Loa loa and Mansonella perstans are not recommended during pregnancy. *Plasmodium spp.* infection status was determined based on a thick blood smears (TBS) made from 10 µl of blood and read by microscopy according to the Lambaréné method (32). Diagnosis of *S. haematobium* infection was based on the detection of parasite eggs in the residue of 10ml urine passed through a Millipore membrane filter, and examined by microscopy. Absence of

infection was confirmed upon three negative results. Blood cell counts and hemoglobin level of the mother was obtained using the ABX Pentra 60 (HORBIA Medical).

Nine milliliters of venous cord blood was drawn after delivery in a heparinized tube. CBMCs were isolated within 24 hours using a Ficoll-Hypaque density gradient centrifugation as described elsewhere (10). CBMC were then fixed with the eBioscience transcription factor fixation and permeabilization kit (eBioscience, San Diego, CA, USA) as per manufacturer instructions.Fixed cells were stored in DMSO freezing medium at −80°C. Staining with fluorescently-labeled antibodies specific to T cell surface markers and to FOXP3, RORγt and Tbet transcription factors was performed for 30 minutes at 4°C . The antibodies used and their combinations are shown in Table 1, whereas the gating strategy is displayed in Figure 1. Data were acquired using a BD FACSCanto II flow cytometer using BD FACSDiva software and analysed using FlowJo.

Table 1: Combinations of monoclonal antibodies used for the flow cytometry analysis

Antibody	Specificity
CD4-V500*/Tbet-PerP5.5#	Tbet positive T cells
CD4-V500*/RoRgT-PE#	RORgT positive T cells
CD4-V500*/CD25-PE-Cy7*/FOXP3-Efluor450#	FOXP3 T-regulatory cells

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IBM SPSS Statistics version 20.0 was used for statistical analysis. Graphs were created using the R graphing package "ggplot2" version 0.9.0. Differences in proportions between the two groups were assessed using the Chi squared test or the Fisher exact test when appropriate. Continuous data were compared with the Student t-test or the Mann-Whitney test when data were not normally distributed. The associations between $CD4^+CD25^{hi}FOXP3^+$ T cells and $CD4^+Tbet^+$ T cells, $CD4^+Ror\gamma T^+$ T cells respectively were analyzed by linear regression analyses. The level of significance was set at 0.05.

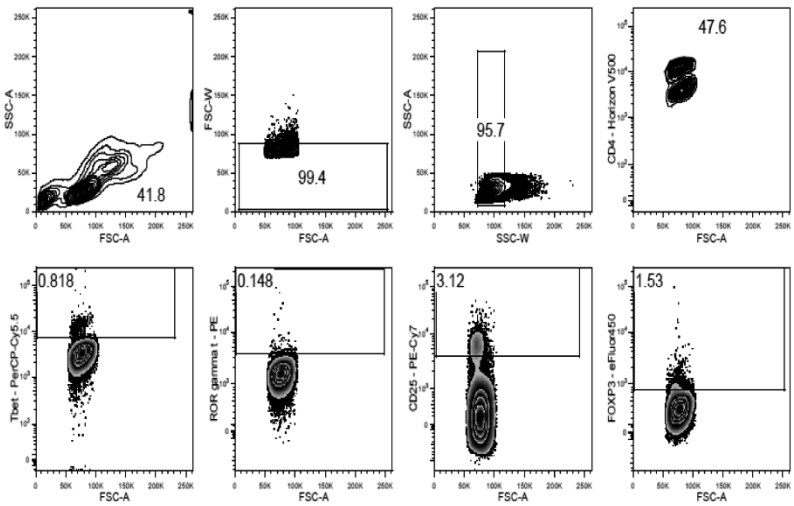

Figure 1: CBMC of neonates from filaria infected and uninfected mothers were isolated, fixed and stained with fluorescently labeled antibodies specific to surface markers (CD4, CD25) and intracellular transcription factors (FOXP3, Tbet, RORγT). Here we show an example of the gating strategy used for the identification of $CD4^{+}Tbet^{+}$, $CD4^{+}ROR\gamma T^{+}$ and $CD4^{+}CD25^{hi}FOXP3^{+}$ T cells. Cells were first gated for lymphocytes (FSC-A vs SSC-A) and for singlets (FSC-A vs FSC-W and SSC-w vs SSC-A). Next, CD4+ cells were selected, to which Tbet, RORgt, CD25 and FOXP3 gating was applied. By combining these gates, Tbet+RORgt-FOXP3-, RORgt+Tbet-FOXP3- and CD25hiFOXP3+Tbet-RORgt- CD4+ T cells could be analysed.

Ethics Statement

The study was approved by the "Comité d'Éthique Régional Indépendant de Lambaréné" (CERIL). The study protocol was explained to each mother and a signed inform consent was sought individually.

Results

A total of 30 pregnant women and their newborns were included in this study. Inclusion was based on the filaria infectious status of the mother so as to have two equally sized groups of filaria infected and uninfected subjects. Both groups were comparable at the time of inclusion with respect to demographic characteristics as shown in Table 2. A total of 13 mothers experienced malaria during pregnancy [5 (53%) were in the group infected with filarial parasites and 8 (33%) in the filarial uninfected group, p = 0,269] but all of them were free of malaria at the time of delivery. Two mothers were found with *S. haematobium* (1 in each group).

Table 2: Baseline characteristic of the mother and the children enrolled in the study

	Microfilaria negative	**Microfilaria positive**	**p**
Number of subjects	15	15	
Characteristic of the mother			
Age in years, median (IQR)	22 (19-28)	23 (21-32)	0.279[#]
Multiparity status, n (%)	15 (100%)	11 (73%)	0.099[##]
Hemoglobin level, mean (±SD)	11.08 (±1.48)	12.27 (±1.71)	0.052[###]
Characteristic of the neonates			
Female, n (%)	7 (47%)	2 (13%)	0.108[##]
Gestational age, median (IQR)	38 (36-40)	39 (37-41)	0.280[#]
Birth weight, median (IQR)	2850 (2470-3130)	2895 (2630-3140)	0.575[#]

[#] *Mann-Withney test* [##] *Fisher exact test* [###] *Independent sample t-test*

Overall the mean percentage of $CD4^+$ T cells in CBMCs was comparable between newborn of filaria infected and uninfected mothers (respectively 47.7 % vs 43.9% of CBMC, p= 0.344). Further characterization of T helper cells based on the signature of transcription factors showed no significant difference between the filaria infected and uninfected groups in the distribution of $CD4^+Tbet^+$ (0.16% vs 0.10%, p= 0.086), CD4+RORγt^+ (0.12% vs 0.14%, p= 0.693) or $CD4^+CD25^{hi}FOXP3^+$ T cells (2.5% of CBMC vs 2.04% respectively, p= 0.210).

In order to assess the association between regulatory T cells and the different T helper subsets, we examined the association between $CD4^+CD25^{hi}FOXP3^+$ T cells and $CD4^+$ $Tbet^+$ T cells, $CD4^+ROR\gamma t^+$ T cells respectively through a linear regression analysis. Analyzing all subjects together, we observed a negative association between Treg and $CD4^+Tbet^+$ T cells (B= -0.149, 95% CI= -0.256 to -0.043, p= 0.008) or $CD4^+ROR\gamma T^+$ T cells (B= -0.175, 95% CI=-0.275 to -0.074, p= 0.001). Interestingly when stratifying our study subjects by their infectious status we observed that the negative association between $CD4^+CD25^{hi}FOXP3^+$ Treg cells and the population of Th1 and Th17 cells was only significant in the offspring of microfilaria infected mothers (Figure2).

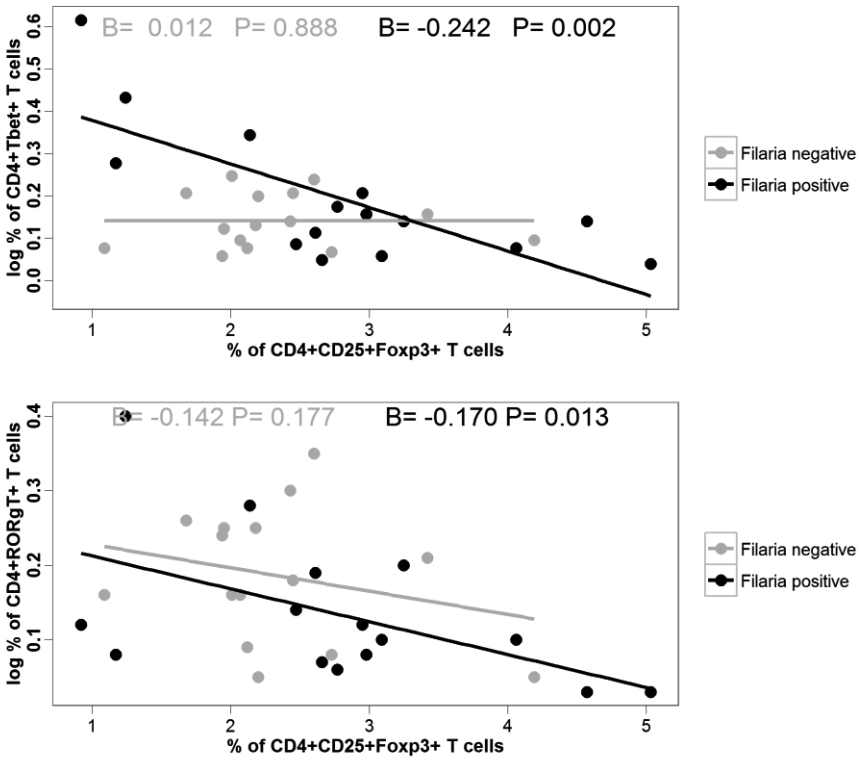

Figure 2: The relation between $CD4^+CD25^{hi}FOXP3^+$ T cells and $CD4^+Tbet^+$ (upper panel) as well as $CD4^+CD25^{hi}FOXP3^+$ T cells and $CD4^+ROR\gamma T^+$ T cells (lower panel) of CBMC of neonates from filaria negative (in grey) and filaria positive (in black) mothers assessed by a linear regression analysis. Each dot shows a single subject while the solid lines represent the regression lines of the model. The strength

of the association between two variables is given by the value of the regression coefficient beta (β) value in each graph. A positive β value indicates a positive association between the variables in the model while a negative β value indicates a negative association. P values are given to indicate the statistical significance of the associations.

Discussion

This exploratory study was designed to assess the effect of maternal filarial infection on the neonatal T helper cells that are known to be involved in malaria driven immune responses, TH1 and TH17, using transcription factors that are now used as hallmarks of T helper cells polarization. To this end we measured the percentage of $CD4^+$ T cells and $CD4^+$ T cells expressing Tbet, RORγt and FOXP3 in CBMCs collected from offspring of filaria infected and uninfected mothers. We did not find a significant effect of maternal filarial infection on the percentage of $Tbet^+$, $ROR\gamma t^+$, $CD25^{hi}FOXP3^+$ $CD4^+$T cells, nor on the level of expression of these transcription factors (data not shown).

Treg cells are important for maintaining immune homeostasis, especially during the course of an infection. Consequently, an expanded Treg compartment has been described in subjects that are chronically infected by parasites such as filarial worms (33). Although, we observed a trend toward an increase of the percentage of Treg cells in CBMC of neonates from filaria infected mothers, this was not statistically significant. When we analyzed how Treg cells were associated with other cell subsets, we observed that they were negatively correlated with Tbet+ and $ROR\gamma t^+$ CD4 T cells. Importantly, this was only seen in the filaria infected group and not in the cells of CBMC of neonates born to uninfected mothers suggesting a stronger functional activity of these cells in infected subjects. In line with our finding, a study reported by Wammes *et al.*, compared both frequency and function of CD4+CD25hiFOXP3+ regulatory T cells in geohelminths infected and uninfected individuals (34). This study found that although the frequency of regulatory T cells was similar between the two groups, their suppressive activity was more pronounced in geohelminths infected subjects (34). Together with our data this result may suggest that activation of CD4+CD25hiFOXP3+ regulatory T cells occur upon exposure of the cells to parasite antigens endowing them with strong functional capacity.

The obvious limitation of our study is the small sample size, which may have prevented the detection of significant differences in percentages of Th1, Th17 and Treg cells in children born to infected and uninfected mothers. Despite this, we could by using a regression model show that in offspring from filaria infected mothers Treg cells could alter effector T cell expansion as described in adults (34–36). When studying regulatory T cells in circumstances where it is not possible to assess their functional capacity (for example in resource poor settings), it might be useful to analyze relationships between their number and outcomes such as other cell subsets that these cells could control or cytokines produced by effector cells.

Altogether our finding that in children born to mothers infected with filarial worms have regulatory T cells that are negatively associated with TH1 or TH17 cells, may have practical implications, as an alteration of effector T cell responsiveness in neonates from helminth infected mothers may lead to a poor immunologic response to vaccines that are usually administered during their first years of life.

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References

1. Bal MS, Mandal NN, Das MK, Kar SK, Sarangi SS, Beuria MK. Transplacental transfer of filarial antigens from Wuchereria bancrofti-infected mothers to their offspring. Parasitology. 2010 Apr; 137 (4):669–73.

2. Seydel LS, Petelski A, van Dam GJ, van der Kleij D, Kruize-Hoeksma YCM, Luty AJF, et al. Association of in utero sensitization to Schistosoma haematobium with enhanced cord blood IgE and increased frequencies of CD5- B cells in African newborns. Am J Trop Med Hyg. 2012 Apr;86(4):613–9.

3. Malhotra I, Dent A, Mungai P, Wamachi A, Ouma JH, Narum DL, et al. Can Prenatal Malaria Exposure Produce an Immune Tolerant Phenotype?: A Prospective Birth Cohort Study in Kenya. PLoS Med. 2009 Jul;6(7).

4. Breitling LP, Fendel R, Mordmueller B, Adegnika AA, Kremsner PG, Luty AJF. Cord Blood Dendritic Cell Subsets in African Newborns Exposed to Plasmodium falciparum In Utero. Infect Immun. 2006 Oct;74(10):5725–9.

5. Malhotra I, Ouma JH, Wamachi A, Kioko J, Mungai P, Njzovu M, et al. Influence of maternal filariasis on childhood infection and immunity to Wuchereria bancrofti in Kenya. Infect Immun. 2003 Sep;71(9):5231–7.

6. Kurtis JD, Higashi A, Wu H-W, Gundogan F, McDonald EA, Sharma S, et al. Maternal Schistosomiasis japonica is associated with maternal, placental, and fetal inflammation. Infect Immun. 2011 Mar;79(3):1254–61.

7. Broen K, Brustoski K, Engelmann I, Luty AJF. Placental Plasmodium falciparum infection: Causes and consequences of in utero sensitization to parasite antigens. Mol Biochem Parasitol. 2007 Jan;151(1):1–8.

8. Adegnika AA, Köhler C, Agnandji ST, Chai SK, Labuda L, Breitling LP, et al. Pregnancy-associated malaria affects toll-like receptor ligand-induced cytokine responses in cord blood. J Infect Dis. 2008 Sep 15;198(6):928–36.

9. Lalor MK, Ben-Smith A, Gorak-Stolinska P, Weir RE, Floyd S, Blitz R, et al. Population differences in immune responses to BCG in infancy. J Infect Dis. 2009 Mar 15;199(6):795–800.

10. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol. 2003 Sep;3(9):733–44.

11. Adegnika AA, Kremsner PG. Epidemiology of malaria and helminth interaction: a review from 2001 to 2011. Curr Opin HIV AIDS. 2012 May;7(3):221–4.

12. Anosike JC, Onwuliri CO, Abanobi OC. A case of probable transplacental transmission of Wuchereria bancrofti microfilariae. Appl Parasitol. 1994 Nov;35(4):294–6.

13. Eberhard ML, Hitch WL, McNeeley DF, Lammie PJ. Transplacental transmission of Wuchereria bancrofti in Haitian women. J Parasitol. 1993 Feb;79(1):62–6.

14. Schwarz NG, Adegnika AA, Breitling LP, Gabor J, Agnandji ST, Newman RD, et al. Placental malaria increases malaria risk in the first 30 months of life. Clin Infect Dis Off Publ Infect Dis Soc Am. 2008 Oct 15;47(8):1017–25.

15. Gallagher M, Malhotra I, Mungai PL, Wamachi AN, Kioko JM, Ouma JH, et al. The effects of maternal helminth and malaria infections on mother-to-child HIV transmission. AIDS Lond Engl. 2005 Nov 4;19(16):1849–55.

16. Soboslay PT, Geiger SM, Drabner B, Banla M, Batchassi E, Kowu LA, et al. Prenatal immune priming in onchocerciasis-onchocerca volvulus-specific cellular responsiveness and cytokine production in newborns from infected mothers. Clin Exp Immunol. 1999 Jul;117(1):130–7.

17. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. J Immunol Baltim Md 1950. 2006 Mar 1;176(5):3248–56.

18. Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, et al. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis. 2012;6(5):e1655.

19. Taylor MD, van der Werf N, Maizels RM. T cells in helminth infection: the regulators and the regulated. Trends Immunol. 2012 Apr;33(4):181–9.

20. Peterson RA. Regulatory T-cells: diverse phenotypes integral to immune homeostasis and suppression. Toxicol Pathol. 2012;40(2):186–204.

21. Grzanka J, Leveson-Gower D, Golab K, Wang X-J, Marek-Trzonkowska N, Krzystyniak A, et al. FoxP3, Helios, and SATB1: Roles and relationships in regulatory T cells. Int Immunopharmacol. 2013 Feb 18;

22. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol. 2007 Mar;8(3):277–84.

23. Chakir H, Wang H, Lefebvre DE, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. J Immunol Methods. 2003 Jul;278(1-2):157–69.

24. Babu S, Kumaraswami V, Nutman TB. Transcriptional Control of Impaired Th1 Responses in Patent Lymphatic Filariasis by T-Box Expressed in T Cells and Suppressor of Cytokine Signaling Genes. Infect Immun. 2005 Jun;73(6):3394–401.

25. Perlaza B-L, Sauzet J-P, Brahimi K, BenMohamed L, Druilhe P. Interferon-γ, a valuable surrogate marker of Plasmodium falciparum pre-erythrocytic stages protective immunity. Malar J. 2011;10(1):27.

26. McCall MBB, Sauerwein RW. Interferon-γ--central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. J Leukoc Biol. 2010 Dec;88(6):1131–43.

27. Bueno LL, Morais CG, Lacerda MV, Fujiwara RT, Braga EM. Interleukin-17 producing T helper cells are increased during natural Plasmodium vivax infection. Acta Trop. 2012 Jul;123(1):53–7.

28. Merlin M, Dupont A, Josse R, Delaporte E, Cheringou H, Garin D, et al. [Epidemiologic aspects of malaria in Gabon]. Médecine Trop Rev Corps Santé Colon. 1990 Mar;50(1):39–46.

29. Akue JP, Nkoghe D, Padilla C, Moussavou G, Moukana H, Mbou RA, et al. Epidemiology of concomitant infection due to Loa loa and Mansonella perstans in Gabon. PLoS Negl Trop Dis. 2011 Oct;5(10):e1329.

30. Nkoghe D, Akue J-P, Gonzalez J-P, Leroy EM. Prevalence of Plasmodium falciparum infection in asymptomatic rural Gabonese populations. Malar J. 2011;10(1):33.

31. Goldsmid JM. Studies on the laboratory diagnosis of human filariasis: Preliminary communication. J Clin Pathol. 1970 Oct;23(7):632–5.

32. Planche T, Krishna S, Kombila M, Engel K, Faucher JF, Ngou-Milama E, et al. Comparison of methods for the rapid laboratory assessment of children with malaria. Am J Trop Med Hyg. 2001 Nov;65(5):599–602.

33. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI, et al. At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. J Immunol Baltim Md 1950. 2010 May 1;184(9):5375–82.

34. Wammes LJ, Hamid F, Wiria AE, de Gier B, Sartono E, Maizels RM, et al. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. Eur J Immunol. 2010 Feb;40(2):437–42.

35. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. J Immunol Baltim Md 1950. 2006 Mar 1;176(5):3248–56.

36. Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, et al. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis. 2012;6(5):e1655.

Chapter 6

Cytokine and chemokine profile of the innate and adaptive immune response of *schistosoma haematobium* and *plasmodium falciparum* single and co-infected school-aged children from an endemic area of Lambaréné, Gabon

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Abstract

Background

Helminths and malaria are among the most prevalent infectious diseases in the world. They both occur in tropical area where they often affect the same populations. There are studies suggesting an effect of helminths on malariometric indices. For example, malaria attacks as well as disease severity has been shown to be influenced by a concurrent chronic helminth infection. However, there are also studies that show no effect of concurrent helminth infections on malarial outcomes. To start addressing this issue, the effect of chronic *Schistosoma haematobium* infection on both the innate and adaptive immune response of *Plasmodium falciparum*-infected subjects was assessed in an area endemic for both these infections in Gabon.

Method

Subjects infected with *S. haematobium* and or *P. falciparum,* as well as a control group with neither of these infections, were recruited. For innate immune response, heparinized blood was obtained and cultured for 24 hours with a panel of TLR ligands. For adaptive immune response, PBMC was isolated and stimulated with SEB for 72 hours. Cytokines and chemokines were measured in supernatants using a multiplex beads array immunoassay. Principal Component analysis was used to assess pattern of cytokine and chemokine responses representing the innate and adaptive components of the immune system.

Results

Overall it was observed that the presence of *P. falciparum* infection was marked by an increase in innate and adaptive immune responsiveness while *S. haematobium* infection was characterized by an increased chemokine profile, with at the same time, lower pro inflammatory markers. When the study subjects were split into single infected and co-infected groups no effect of *S. haematobium* on the immune response of *P. falciparum* infected subjects was observed,

neither for the innate nor for the adaptive component of the immune response.

Conclusion

This study provides original information on the cellular immune response of *S. haematobium* and/or *P. falciparum* in infected subjects. It rules out an effect of *S. haematobium* on the cytokine profile of subjects co-infected with *P. falciparum*.

Keywords

Malaria, Schistosomiasis, Co-infection, Innate immune response, Adaptive immune response, Principal component analysis, Epidemiology, Lambaréné, School-aged children

Background

In *Plasmodium spp*. infected subjects the ability to control the development of the parasite depends largely on the balance between pro and anti-inflammatory mediators of their immune response [1,2]. Acute *Plasmodium falciparum* infection is usually associated with an increase of INFγ and TNF, regarded as the markers of the Th1 and pro-inflammatory response [2,3]. This pro-inflammatory response is thought to be needed to impede the multiplication of the parasite and favour its clearance, both in human and animal models [2-4]. While important for parasite clearance a powerful Th1 and pro-inflammatory response could also be detrimental for the host if uncontrolled, leading to tissue damage and severe disease [5,6]. This is supported by the importance of the anti-inflammatory network characterized by an expansion of the regulatory T cells [7-10], as well as by the activation of negative regulators like the CTLA4 or PD-1, transmembrane receptors, during malaria infection [11,12]. Moreover, as Th1 responses can be counteracted by Th2 cells, the presence of a strong Th2 response might also influence anti-malarial immunity.

In areas where malaria is endemic, it is the norm that *Plasmodium*-infected people also suffer from a concurrent helminth infection [13,14]. Helminths have repeatedly been shown to modulate the immune system of their host in order to survive [15]. Chronic helminthiasis is usually characterized by a marked Th2 response [16,17] as well as by the induction of a regulatory network [18,19] that could consequently impair the host immune response to other antigens [20]. Whether a concurrent helminth infection of the host can affect his immune response to *Plasmodium spp.* co-infection is still debated [21,22]. Population-based studies conducted to assess the effect of helminths on malariometric indices and on the immune response of *P. falciparum* infected subjects have so far revealed contrasting results. For example, in Senegal, Sokhna *et al.* observed that children with *Schistosoma mansoni* had an increased incidence of clinical malaria in comparison to their uninfected counterparts [23], while in Mali, Lyke and colleagues reported a protective effect of *S. haematobium* infection against malaria [24]. A similar divergent picture has also emerged when considering the cellular response of malaria and helminth co-

infected subjects. For example in Senegal, Diallo *et al.* reported a significant increase of the plasma concentration of TNF and IFNγ measured in *S. haematobium* and *P. falciparum* co-infected children in comparison to their *P. falciparum* single infected counterpart [25]. In the same studies, they also observed a significant increase of the plasma concentration of TNF, IFNγ, IL-10, TGF-β, sTNF-RI and sTNF-RII rates in co-infected subjects [25]. Similarly in Ghana, Hartgers *et al.* compared the cytokine response of *S. haematobium* subjects to uninfected ones when their whole blood were stimulated with *P. falciparum* infected red blood cells (iRBCs) and observed that the measured level of IL-10 was significantly higher in the infected group [26]. Inversely, in Mali Lyke *et al.* reported a decreased level of IL-10 in plasma from *S. haematobium* and malaria co-infected subjects by comparison to malaria only subjects [27].

Some reports have suggested that a concurrent helminth infection is associated with elevated cytokines in particular pro inflammatory ones compared to *P. falciparum* infected subjects [25,26], while in others either no effect or even a decreased in these cytokines [27,28,29]. It is important to note that each of these studies assessed the immune system of infected people from a different angle, either by using different stimuli or by characterizing a different cells type. Moreover none has yet attempted to provide information on how helminths affect both the innate and the adaptive immune response of *P. falciparum*-infected subjects within the same cohort.

This study provides information on the cellular immune response of *P. falciparum*-infected subjects, with or without concurrent *S. haematobium* infection. Instead of assessing cytokines responses individually, a more global approach was taken to profile the pattern of cytokine responses in the study subjects. The study hypothesis was that a comprehensive and integrative assessment of multiple cytokines involved in the innate or the adaptive immune response of co-infected subjects would provide a better insight into the effect of *S. haematobium* on the immune response of *P. falciparum* infected subjects.

Methods

Recruitment of study participants and diagnosis of parasitic infections

This study was cross sectional and was conducted in the Bindo village located in the Moyen-Ogooué province in Gabon. The Bindo village is endemic for both *S. haematobium* and malaria [30]. School children from six to 16 years of age, attending the only school of the village were included. Urine and blood samples were collected at inclusion for the diagnosis of *S. haematobium* and *P. falciparum* infection as well as for immunological assays. A thorough description of the parasitological test and the immunological assays has been previously published in a study protocol article [31]. Briefly, *S. haematobium* infection was determined by the detection of eggs by microscopy in 10 ml of filtrated urine. *Schistosoma haematobium*-uninfected subjects were those who did not show any eggs in three samples of urine collected on three consecutive days. Detection of *P. falciparum* infection was made by real time-PCR performed on DNA extracted from EDTA blood pellet kept frozen in - 80°C [32].

Immunological assays

Peripheral blood was collected in sodium heparinized tubes for every child as described elsewhere [31]. To assess the innate immune response heparinised blood was diluted in RPMI (1:1) and cultured for 24 hours at 37°C with a panel of five different Toll like receptor ligands (LPS [TLR4], PAM3 [TLR1/2], CPG [TLR9] CL097 [TLR7/8]), *S. haematobium* eggs antigens (SEA) and a combination of LPS and SEA. Supernatant was collected after 24 hours and cytokine production was measured using the multiplex beads array immunoassay. The cytokines/chemokines quantified for this ex-vivo assay were IFNα2, IL-1ß, IL-6, IL-10, IL-12p70, IL-13, IFNγ, MCP-1, MIP-1α, MIP-1ß, TNF and IP-10.

In order to assess the adaptive immune response, PBMC were isolated by density gradient centrifugation on Ficoll as already described [31]. PBMCs were cultured for 72 hours at 37°C with SEB and supernatant

was collected for the measurement of a panel of 11 different cytokines (TNF, IFNγ, IL-2, IL-4, IL-5, IL-13, IL-17A, IL-17 F, IL-22, IL-10, and IL-21) by a multiplex beads array immunoassay.

Statistics

Chi square and fisher's exact test were used to compare categorical variables. Not normally distributed quantitative variables were transformed either by Log10 or Box-Cox transformation. Student *t*-test and ANOVA were performed when data met the assumption of normality. Otherwise the non-parametric Mann Whitney and Kruskal Wallis tests were used. Correlation between two continuous variables was assessed using the spearman rho test.

Principal component analysis (PCA) was carried out on the cytokine variables in order to summarize them. Of note PCA is a mathematical technique that allows reducing the dimension of large dataset by identifying new summary variables also called principal component (PC). Each principal component is made of a set of original variables that share a certain level of correlation. All the analysis was performed on medium subtracted data. Negative values were set to zero. Cytokine from the innate [obtained after whole blood stimulation] and the adaptive panels [obtained after PBMC stimulation] were analyzed independently by PCA. As mentioned above the innate panel consisted of 12 cytokines measured after stimulation of cells by six different conditions. Before performing the PCA an average cytokine response were calculated for each subject by taking the mean of the cytokine level obtained with the six different stimuli. This step only concerned the innate panel and was not needed for cytokine of the adaptive panel since the cytokine response to one stimulus was only assessed. All variables were transformed either using a log10 or a Box-Cox transformation to reduce skewness prior to PCA. Principal components identified were considered for further analysis if their eigenvalues were above one. No rotation was applied. Individuals PC scores were obtained for each subject and used for comparison between groups. All the statistical tests were computed using R version 3.0.1. The R packages ggplot2 version 0.9.3.1 and FactoMineR version 1.25 were used for making graphs and performing the PCA respectively. Statistical significance was set for p value below 0.05.

Ethics

The study was approved by the "Comité d'éthique Régional de Lambaréné" (CERIL). Informed consent was obtained from parents or legal guardians of each of the children included in the study. Appropriate treatment was given to children found with *P. falciparum* or *S. haematobium* infection as per the local guidelines.

Results

Characteristics of the study subjects

The recruitment of the study participants took place in May 2011. Overall 125 subjects aged from six to 16 years were included. Among them 63 (50.4%) were infected with *S. haematobium* while 66 (53%) carried *P. falciparum* in their blood as determined by PCR. When considering co-infection four different groups were compared as shown in Table 1. Children with *P. falciparum* single infection were younger and had a lower haemoglobin level. No other significant differences were found between the different groups.

Table 1 Characteristics of the study subjects divided by infection status

	S.h-/ P.f-	*S.h +/ P.f-*	*S.h -/ P.f+*	*S.h +/ P.f+*	p-value
Number of subjects (%)	28 (22%)	31 (25%)	34 (27%)	32 (26%)	-
Gender: M/F	14/14	13/18	19/15	18/14	0.63
Age in years: Median (IQR)	11 (3.25)	11(5)	9.5(4)	13(3)	0.02
Weight in kgs: Median(IQR)	31.5(12.25)	35(15)	30(9.8)	36(18.25)	0.05
Haemoglobin in g/dl: Median (IQR)	11.9(0.8)	12.25(0.95)	11.1(1.08)	11.8(1.6)	0.003
Number of subjects living in the village for more than 5 years (%)	17(61%)	21(75%)	15(44%)	19(61%)	0.10
Number of filaria infected subjects (%)	2(7%)	4(13%)	2(6%)	2(6%)	0.67
Number of subjects previously treated for ***S. haematobium***: (%)	9(32%)	21(41%)	7(21%)	14(45%)	0.16
S. haematobium eggs count per 10 ml : Median (IQR)	0	15(49.5)	0	52.5(88.5)	0.26[#]
P. falciparum CT value Median (IQR)	0	0	28.9 (7.3)	30.7 (8.6)	0.25[#]

Innate immune responses

Cytokine and chemokines were measured following the stimulation of whole blood with a panel of TLR ligands for 24 hours. In Figure 1a, the levels of measureable chemokines/cytokines are shown. The chemokines/cytokines showed a certain degree of correlation (Figure 2a) that prompted us to perform a principal component analysis. The PCA identified two PCs (respectively named innate PC1 (iPC1) and iPC2) that summarized the 12 measured chemokines/cytokines. These PCs are described in Table 2. Briefly the iPC1 comprised of almost all chemokines/cytokines included in the PC analysis and, therefore, was interpreted as reflecting the general responsiveness. The iPC2 was best characterized by four chemokines/cytokines that clustered into two groups; the MCP1-MCAF/MIP-1β, which were positively loaded and the INFγ/TNF, which were negatively loaded in the PCs. In other words an increase of PC2 would represent an increase of MCP1-MCAF/MIP-1α and a simultaneous decrease of INFγ/TNF.

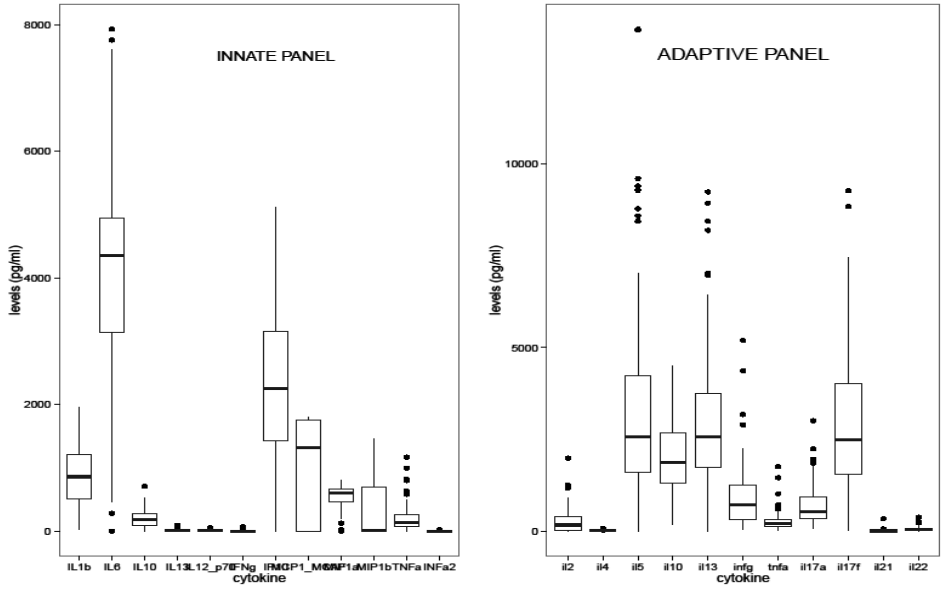

Figure 1: Levels of the cytokines measured for the innate (left) and the adaptive (right) panels. For the innate panel the mean response was calculated per cytokine for the 6 different antigens that were used cin the whole blood assay. This step was not needed for cytokine pertaining to the adaptive panel since only the cytokine response after SEB stimulation was assessed. Boxes represent the magnitude of the overall response of the study subjects per each cytokine. Whiskers represent minimal and maximal concentrations and dots are indicative of subjects with outlier values.

Neither age nor gender affected iPC1 or iPC2. In a univariate analysis, iPC1 was higher in *P. falciparum* infected subjects in comparison to subjects with no *P. falciparum* infection (median level of the iPC1 scores: 0.133 in infected *vs* 0.003 in uninfected subjects, p =0.019, Figure 3a), whereas *S. haematobium* infection was associated with an increased iPC2 (0.6 in infected vs 0.04 in uninfected, p = 0.016, Figure 3b). This indicates that during *P. falciparum* infection there is an enhancement of responses to innate stimuli in general while *S. haematobium* infection appears to lead to a selective increase in the release of macrophage-released chemokines, and at the same time to a decrease of pro-inflammatory cytokines in response to TLR stimuli.

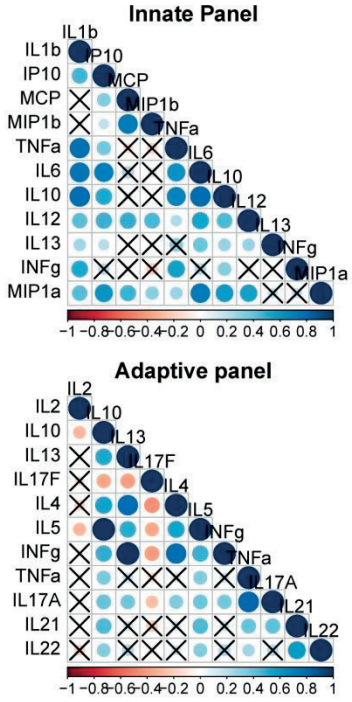

Figure 2 Correlation matrix of the cytokines of the innate (upper) and the adaptive (lower) panels. The pair wise correlation between the different cytokines measured is depicted. The intensity of the colours as well as the diameter of the circles give an indication of the degree of correlation between two cytokines and reflect the strength of spearman's rho correlation coefficient. The crosses represent correlation coefficients that were not statistically significant. Significance was tested using a spearman rank test and level of significance was set at $p < 0.05$.

To further assess immune response in single and co-infected subjects with particular emphasis on the question whether *S. haematobium* affects response associated with *P. falciparum,* the study population was divided into four groups of uninfected, infected with *P. falciparum* only, *S. haematobium* only and infected with both. What was observed was that iPC1 was higher in those with *P. falciparum* infection, and statistically significantly so in those co-infected with *S. haematobium* (Table 3). These data indicate that *P. falciparum* effect on the immune system is not influenced by concurrent *S. haematobium* infection.

Table 2: Description of the different principal components identified for the innate panel (iPC1 and iPC2) and the adaptive panel (aPC1, aPC2 and aPC3)

Cytokines/ Chemokines	iPC1		iPC2		aPC1		aPC2		aPC3	
	Score	Contribution	Score	Contribution	Score	Contribution	Score	Contribution	Score	Contribution
IL1b	0.4	14	−0.2	6	-	-	-	-	-	-
IP10	0.3	11	-	-	-	-	-	-	-	-
MCP1-MCAF	0.2	2	0.5	27	-	-	-	-	-	-
MIP1a	0.3	12	0.2	5	-	-	-	-	-	-
MIP1b	0.1	1	0.5	29	-	-	-	-	-	-
TNF	0.3	10	−0.4	13	-	-	-	-	-	-
IL6	0.4	17	-	-	-	-	-	-	-	-
IL10	0.4	14	−0.1	2	-	-	-	-	-	-
IL12	0.3	9	0.3	8	-	-	-	-	-	-
IL13	0.2	4	-	-	-	-	-	-	-	-
INFγ	0.2	4	−0.3	10	-	-	-	-	-	-
IL5	-	-	-	-	0.34	11.7	0.55	29.8	−0.13	1.7
IL10	-	-	-	-	0.39	15.1	0.21	4.6	−0.03	0.1
IL13	-	-	-	-	0.35	12.6	0.49	23.7	−0.18	3.4
INFγ	-	-	-	-	0.37	13.7	−0.24	5.6	0.56	31
TNF	-	-	-	-	0.42	17.6	−0.09	0.9	0.49	24.4
IL17A	-	-	-	-	0.36	13.2	−0.31	9.8	−0.13	1.6
IL21	-	-	-	-	−0.31	9.3	0.32	10.8	0.42	17.6
IL22	-	-	-	-	−0.26	6.9	0.38	14.5	0.45	20.23

Adaptive immune responses

As TLRs stimulate the innate immune system in general, to assess the general response of the adaptive immune system the PBMC were stimulated with SEB which is a superantigen capable of triggering a

polyclonal T cells activation, part of the general adaptive immune responsiveness. The cytokines measured are shown in Figure 1b and the extent of their correlation in Figure 2b. To profile the cytokine response, a PCA was performed. As shown in Table 2, three different PCs (adaptive PC1 (aPC1), aPC2 and aPC3) were identify. Based on the type of cytokines that contributed to the PC, they were interpreted as follows: aPC1 represented the general immune responsiveness; aPC2 the Th2/Th17; and aPC3 Th1/Th17 response.

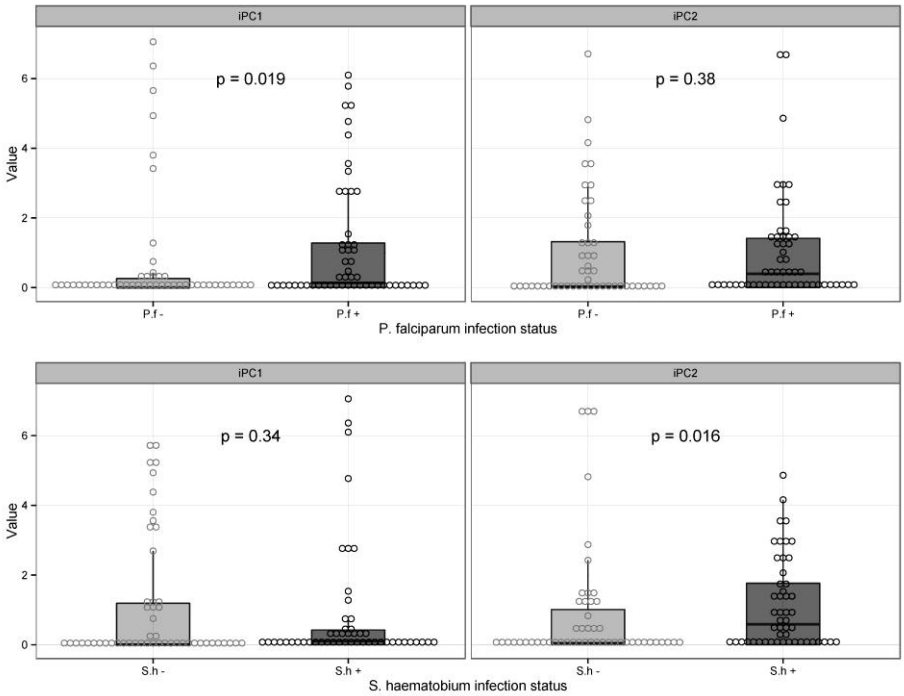

Figure 3 Effect of *P. falciparum* and *S. haematobium* single infection or coinfection on the levels of the principal components reflecting the innate immune response of the study subjects. Two principal components (iPC1 and iPC2) were identified and explained 67% of the variance in the database. The iPC1 was made of almost all the cytokine included in the model and thus was representative of the innate immune responsiveness of the study subjects. The iPC2, in contrast, was formed by 4 cytokines who clustered into two groups MCP1-MCAF and MIP1b positively loaded in the iPC2 and INFγ and TNF that were negatively loaded. Thus an increase of the iPC2 will mirror an increase of the positively loaded chemokines and a decrease of the negatively loaded cytokines. The box plots represent the median and the interquartile range of the different iPCs while the whiskers show the minimal and maximal value.

There were no differences between males and females for the aPC1, aPC2, and aPC3 (data not shown). A strong correlation between aPC1

and the age of the study participants (rho = 0.3, p < 0.008) was observed but no effect of age was seen on aPC2 (rho = 0.1, p = 0.3) or aPC3 (rho = −0.1, p = 0.4). As shown in Figure 4a, a trend toward an increase of the aPC1 was seen with *P. falciparum* infection but no effect was observed on the aPC2 or aPC3. Moreover none of the components, aPC1, aPC2 or aPC3 was affected by *S. haematobium* infection (Figure 4b). Finally, no statically significant differences between groups were detected when *P. falciparum* and *S. haematobium* co-infected subjects were compared with those with single or no infection (Table 3).

Table 3: Effect of *P. falciparum* (*P.f.*) and *S. haematobium (S.h)* co-infection on the different principal components identified from the innate (iPC) and the adaptive (aPC) immune response

	S.h-/P.f- **Median (IQR)**	***S.h* +/ *P.f* -** **Median (IQR)**	***S.h* -/ *P.f* +** **Median (IQR)**	***S.h* +/ *P.f* +** **Median (IQR)**	**p-value**
iPC1	0 (0–0.03)	0.05 (0–0.31)	0.24 (0–1.28)	0.13(0.005-0.94)	0.046[#]
iPC2	0 (0–0.31)	0.73(0–2.37)	0.12 (0–1.22)	0.53 (0.005-1.48)	0.047[##]
aPC1	0.26 (0.11- 0.89)	0.38 (0.05 – 1.25)	0.36 (0.11 – 1.4)	1 (0.18 – 3.31)	0.22
aPC2	0.53 (0.18 – 0.84)	0.66 (0.2-1.26)	1.27 (0.4-1.7)	0.75 (0.2 -1.6)	0.42
aPC3	0.6 (0.25 – 1.33)	0.47 (0.1-1.6)	1.34 (0.24-3.019)	0.25 (0.05-1.1)	0.56

S.h-/ P.f- : Subjects not infected by either *S. haematobium* or *P. falciparum. S.h+/ P.f-*: Subjects with single *S. haematobium* infection. *S.h-/ P.f+*: Subjects with single *P. falciparum* infection. *S.h+/ P.f+*: *S. haematobium* and *P. falciparum* co-infected subjects. M: male and F: female. CT value represents the value of the cycle threshold. [#] Two by two comparison of the groups are shown and show significant difference between *S.h-/P.f-* vs *S.h +/ P.f +* (p = 0.04). [##] Two by two comparison show significant difference between *S.h-/P.f-* vs *S.h +/ P.f –* and between *S.h-/P.f-* vs *S.h +/ P.f +* (p = 0.03 and 0.02 respectively).

Discussion

The main objective of this study was to determine whether chronic *S. haematobium* infection was able to affect the cellular immune response of *P. falciparum* infected subjects. By measuring the cytokine production after in-vitro stimulation, the innate and adaptive immune responses of the study subjects were profiled. Here, rather than assessing single cytokines, the pattern of cytokine responses of the study subjects using a PCA was evaluated. PCA is a mathematical tool widely used in the field of biology. It has the advantage of summarizing highly correlated variables in new latent and synthetic variables called principal components that can unveil new pattern of responses [33, 34].

Two PCs (iPCs) that summarize the innate cytokine responses of the study participants were identify as well as 3 PCs (aPCs) for the adaptive cytokine responses. The interpretation of these different PCs shows that cytokines are released with a certain degree of correlation.

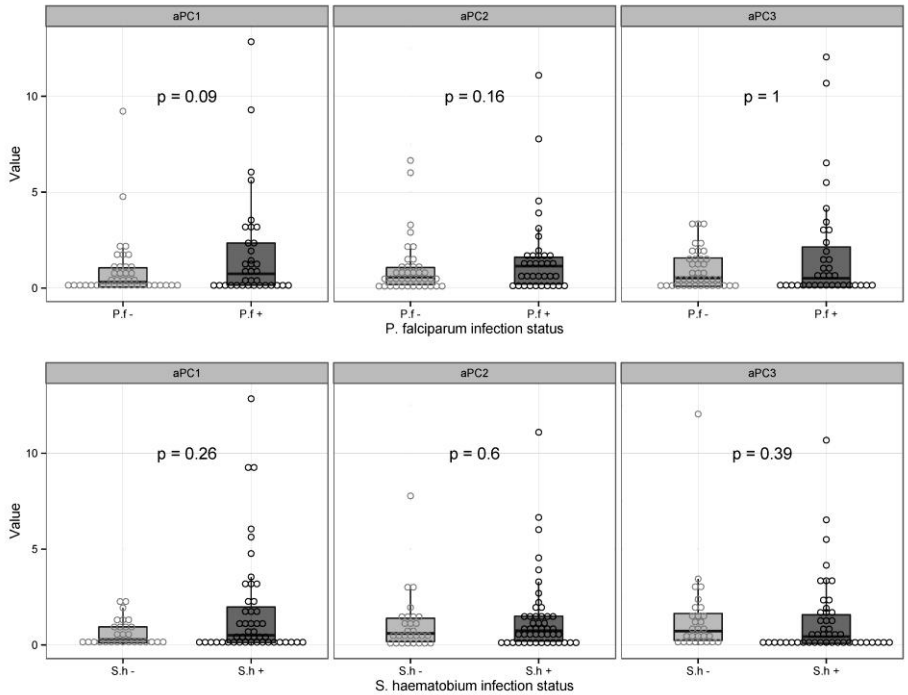

Figure 4: Effect of *P. falciparum* and *S. haematobium* single infection or coinfection on the levels of the principal components reflecting the adaptive immune response of the study subjects. Three different principal components were identified and explained 76% of the variance in the dataset. The aPC1 was formed by almost all the cytokines included in the model and thus was representative of the adaptive immune responsiveness of the study subjects. The aPC2 and aPC3 was representative of the Th2/Th17 and Th1/Th17 respectively. They were all positively loaded on their respective PCs. The box plots represent the median and the interquartile range of the different iPCs while the whiskers show the minimal and maximal value.

This is supported by the fact that none of the PCs identified was made of only one cytokine and at least two cytokines were represented in every PC. Moreover, it was noticed that within the same PC cytokines were either negatively or positively correlated. For example in the iPC2, Th1 type cytokines (IFNγ and TNF) were negatively correlated with cytokines released by macrophages (MCP1-MCAF and MIP1β) implying an antagonistic effect that may need further investigations.

In a number of studies it has been shown that *S. haematobium* infection can influence the innate immune response of the human host. For instance in population based studies, schistosomiasis has been linked with functional impairment of human myeloid dendritic cells [35] and their response to TLR ligands [36-38]. *Schistosoma haematobium* excretory-secretory products can prime dendritic cells to shape the adaptive response toward a Th2 phenotype [38,39]. While this immune profile is thought to limit the damage caused by schistosomes in the human host, it could alter the host immune response to a concurrent *P. falciparum* co-infection. What was observed in this study is that *P. falciparum* infection was marked by an increase of the iPC1 and aPC1, which represented the innate and adaptive general immune responsiveness. Interestingly, this was not the case for *S. haematobium* infection, which was associated with an increased level of chemokines (MCP1-MCAF and MIP1b) and the decrease of pro-inflammatory cytokines, namely INFγ and TNF. This indicates that the immune system responds differently to *P. falciparum* and to *S. haematobium* infection. In *P. falciparum*-infected subjects the increase of the iPC1 and aPC1 component is in line with the immune profile seen in asymptomatic *P. falciparum* infected subjects [40]. This is also in line with the literature indicating that in subjects chronically infected with *S. haematobium* there is a down modulation of the pro inflammatory response that is thought to allow the survival of the parasites [18,19]. These observations regarding *S. haematobium* are in line with results of two independent studies that assessed the innate immune response of schistosome-infected subjects. In the first study, Turner *et al.* observed that upon stimulation of whole blood with schistosome excretory/secretory products, *S. haematobium* infected subjects had an enhanced production of IL-10, an anti inflammatory cytokine, whereas the level of the pro-inflammatory cytokine TNF was not different from the uninfected subjects [41]. In the second study, Van der Kleij *et al.* observed that *S. haematobium* infection was associated with a significant decrease in responsiveness to LPS irrespective of pro or anti inflammatory cytokines [37]. However, a study by Meurs *et al.* reported that PBMC of *S. haematobium* infected subjects produced significantly more TNF after stimulation with Pam3 a TLR2/1 ligand in comparison to their *S. haematobium* uninfected counterparts [36]. These differences are difficult to reconcile but the culture methods [whole blood versus PBMC], seasonal fluctuation in immune

responses, or other factors such as different environments or co infections [42,43], need to be taken into consideration when comparing studies.

This study did not observe an effect of *S. haematobium* on the innate and adaptive cytokine profile of *P. falciparum* infected subjects. The current body of evidence on helminth and malaria co-infection and its effect on the host immune response has so far given contrasting results. For example a cross sectional study showed no impact of light intensity Ascaris infection on the immune response of malaria infected subjects [44]. In a study conducted in Mali, *S. haematobium* infected and uninfected subjects were followed up until the time to the first malaria episode and serum cytokines were measured at the time of inclusion and at the time when study subjects became infected with *P. falciparum* [27]. At baseline the level of IL-4, IL-6, IL-10 and IFNγ cytokines were all higher in subjects infected with *S. haematobium* by comparison to uninfected subjects. However, when these participants developed an acute episode of malaria IL-6 and IL-10 cytokines increased considerably in all groups, but to a higher extent in subjects who were free of schistosome infection [27], which would suggest that *S. haematobium* impedes the cytokine storm. It has to also be noted that, looking at the results differently, which is that at the time of malaria infection, the baseline differences in IL-6 and IL-10 in the *S. haematobium* infected and uninfected subjects, fell short of statistical significance, one might conclude that there is no difference between subjects with single malaria versus those who were coinfected. In contrast, in a study in Senegal, where *P. falciparum* infected participants were compared to *S. haematobium* and *P. falciparum* co-infected subjects; Diallo *et al.* reported that the plasma concentration of IL-10, TGFβ, INFγ (but not INFα) was higher in co-infected subjects than in those with single infection. The same authors, when examining *in vitro* production of cytokines by mononuclear cells stimulated with *P. falciparum* schizont extracts and MSP1-19 antigens reported an increase of IL-10 and INFγ but not TGFβ, IL-12 or IL-13 in subjects with *P. falciparum* infection compared with subjects co-infected with *P. falciparum* and *S. haematobium* [45]. Finally, a study conducted by Hartgers *et al.* in Ghana showed higher response to malaria antigens in terms of IL-10 but not INFγ, IL-6, TNF in helminth infected subjects in comparison to those free of helminth infection [26]. It is important to

emphasize that in the study of Hartgers *et al.,* the response to malaria antigens was compared between *S. haematobium*-infected and uninfected subjects and, therefore, malaria infection was artificially mimicked by the use of antigens from *P. falciparum*. Regarding, the Senegal studies, the *P. falciparum* singly infected individuals originated from a village where *S. haematobium* infection was never reported before, whereas co-infected subjects were from an entirely different village endemic for both *S. haematobium* and *P. falciparum.* Therefore, it is possible that the differences reported, mirror the exposure to different environmental factors rather than to *S. haematobium*. This is supported by the work by Smolen and colleagues who compared the immune response of children across four different continents. Using a standardized procedure they observed considerable heterogeneity in the cytokine responses in the different geographical areas [42].

One obvious limitation of the present study is that it is cross sectional and one could argue that it does not provide information on history of past helminth infections that are capable of imprinting the host immune system. For example, the innate immune system has been shown to be able to keep a "memory" of early exposure to PAMPs through a process called "trained immunity" which is not addressed in this study [46]. Additional limitation concerns the sample size of the study that may not be sufficient to detect an effect of helminths on *P. falciparum* modulated immune responses. However, this study was carried out in a relatively small community where it was possible to enroll all the school-aged children willing to participate and fulfilling the inclusion criteria. Despite these limitations the present study provides original information on the cellular immune response of *S. haematobium* and/or *P. falciparum* infected subjects. It showed that *P. falciparum,* but not *S. haematobium,* infection was associated with an increase of the immune responsiveness of the study subjects but it did not evidenced an effect of *S. haematobium* on the immune response that were measured in the *P. falciparum*-infected participants.

Conclusions

This study assessed the effect of *S. haematobium* on the pattern of cytokine responses elicited in subjects concurrently infected with *P. falciparum*. It shows that *P. falciparum* infection is associated with an increased immune responsiveness which is not affected by *S. haematobium* co-infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

UAN, JFZ, RFKK carried on the study on the field. They were responsible of the screening and the enrolment of the study participants. UAN carried on the different immunological assays, performed the statistical analysis and wrote the first draft. AAA, MML and BM advise on the epidemiological aspect of the study. HS advise on the immunological aspect of the study. PGK, MY and AAA designed and coordinated the study. All authors participated in the manuscript preparation, read and approved the final version of the manuscript.

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References

1. Clark IA, Alleva LM, Budd AC, Cowden WB. Understanding the role of inflammatory cytokines in malaria and related diseases. Travel Med Infect Dis. 2008;6:67–81.

2. Winkler S, Willheim M, Baier K, Schmid D, Aichelburg A, Graninger W, et al. Reciprocal regulation of Th1- and Th2-cytokine-producing T cells during clearance of parasitemia in *Plasmodium falciparum* malaria. Infect Immun. 1998;66:6040–4.

3. Clark IA, Budd AC, Alleva LM, Cowden WB. Human malarial disease: a consequence of inflammatory cytokine release. Malar J. 2006;5:85.

4. Perlaza B-L, Sauzet J-P, Brahimi K, BenMohamed L, Druilhe P. Interferon-γ, a valuable surrogate marker of *Plasmodium falciparum* pre-erythrocytic stages protective immunity. Malar J. 2011;10:27.

5. Palomo J, Fauconnier M, Coquard L, Gilles M, Meme S, Szeremeta F, et al. Type I interferons contribute to experimental cerebral malaria development in response to sporozoite or blood-stage *Plasmodium berghei* ANKA. Eur J Immunol. 2013;43:2683–95.

6. Villegas-Mendez A, Greig R, Shaw TN, de Souza JB, Gwyer Findlay E, Stumhofer JS, et al. IFN-γ-producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T cell accumulation within the brain. J Immunol. 2012;189:968–79.

7. Nie CQ, Bernard NJ, Schofield L, Hansen DS. CD4+ CD25+ regulatory T cells suppress CD4+ T-cell function and inhibit the development of *Plasmodium berghei*-specific TH1 responses involved in cerebral malaria pathogenesis. Infect Immun. 2007;75:2275–82.

8. Haque A, Best SE, Amante FH, Mustafah S, Desbarrieres L, de Labastida F, et al. CD4+ natural regulatory T cells prevent experimental cerebral malaria via CTLA-4 when expanded in vivo. PLoS Pathog. 2010;6:e1001221.

9. Finney OC, Nwakanma D, Conway DJ, Walther M, Riley EM. Homeostatic regulation of T effector to Treg ratios in an area of seasonal malaria transmission. Eur J Immunol. 2009;39:1288–300.

10. Walther M, Tongren JE, Andrews L, Korbel D, King E, Fletcher H, et al. Upregulation of TGF-beta, FOXP3, and CD4 + CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. Immunity. 2005;23:287–96.

11. Hafalla JCR, Claser C, Couper KN, Grau GE, Renia L, de Souza JB, et al. The CTLA-4 and PD-1/PD-L1 inhibitory pathways independently regulate host resistance to Plasmodium-induced acute immune pathology. PLoS Pathog. 2012;8:e1002504.

12. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, et al. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. Nat Immunol. 2012;13:188–95.

13. Brooker SJ, Pullan RL, Gitonga CW, Ashton RA, Kolaczinski JH, Kabatereine NB, et al. Plasmodium-helminth coinfection and its sources of heterogeneity across East Africa. J Infect Dis. 2012;205:841–52.

14. Adegnika AA, Kremsner PG. Epidemiology of malaria and helminth interaction: a review from 2001 to 2011. Curr Opin HIV AIDS. 2012;7:221–4.

15. Hartgers FC, Yazdanbakhsh M. Co-infection of helminths and malaria: modulation of the immune responses to malaria. Parasite Immunol. 2006;28:497–506.

16. Grzych JM, Pearce E, Cheever A, Caulada ZA, Caspar P, Heiny S, et al. Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. J Immunol. 1991;146:1322–7.

17. De Oliveira Fraga LA, Torrero MN, Tocheva AS, Mitre E, Davies SJ. Induction of type 2 responses by schistosome worms during prepatent infection. J Infect Dis. 2010;201:464–72.

18. Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, et al. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis. 2012;6:e1655.

19. Nausch N, Midzi N, Mduluza T, Maizels RM, Mutapi F. Regulatory and activated T cells in human *Schistosoma haematobium* infections. PLoS One. 2011;6:e16860.

20. Sabin EA, Araujo MI, Carvalho EM, Pearce EJ. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with *Schistosoma mansoni*. J Infect Dis. 1996;173:269–72.

21. Nacher M. Interactions between worms and malaria: good worms or bad worms? Malar J. 2011;10:259.

22. Metenou S, Babu S, Nutman TB. Impact of filarial infections on coincident intracellular pathogens: *Mycobacterium tuberculosis* and *Plasmodium falciparum*. Curr Opin HIV AIDS. 2012;7:231–8.

23. Sokhna C, Hesran J-YL, Mbaye PA, Akiana J, Camara P, Diop M, et al. Increase of malaria attacks among children presenting concomitant infection by *Schistosoma mansoni* in Senegal. Malar J. 2004;3:43.

24. Lyke KE, Dicko A, Dabo A, Sangare L, Kone A, Coulibaly D, et al. Association of *Schistosoma haematobium* infection with protection against acute *Plasmodium falciparum* malaria in Malian children. Am J Trop Med Hyg. 2005;73:1124–30.

25. Diallo TO, Remoue F, Schacht AM, Charrier N, Dompnier J-P, Pillet S, et al. Schistosomiasis co-infection in humans influences inflammatory markers in uncomplicated *Plasmodium falciparum* malaria. Parasite Immunol. 2004;26:365–9.

26. Hartgers FC, Obeng BB, Kruize YCM, Dijkhuis A, McCall M, Sauerwein RW, et al. Responses to malarial antigens are altered in helminth-infected children. J Infect Dis. 2009;199:1528–35.

27. Lyke KE, Dabo A, Sangare L, Arama C, Daou M, Diarra I, et al. Effects of concomitant *Schistosoma haematobium* infection on the serum cytokine levels elicited by acute *Plasmodium falciparum* malaria infection in Malian children. Infect Immun. 2006;74:5718–24.

28. Lyke KE, Dabo A, Arama C, Daou M, Diarra I, Wang A, et al. Reduced T regulatory cell response during acute *Plasmodium falciparum* infection in Malian children co-infected with *Schistosoma haematobium*. PLoS One. 2012;7:e31647.

29. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly YI, Diallo AA, et al. Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol. 2011;186:4725–33.

30. Adegnika AA, Zinsou JF, Issifou S, Ateba-Ngoa U, Kassa RF, Feugap EN, et al. Randomized, controlled, assessor-blind clinical trial to assess the efficacy of single- versus repeated-dose albendazole to treat *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm infection. Antimicrob Agents Chemother. 2014;58:2535–40.

31. Ngoa UA, Zinsou JF, Kassa RFK, Feugap EN, Honkpehedji YJ, Massinga-Loembe M, et al. Assessment of the effect of *Schistosoma haematobium* co infection on malaria parasites and immune responses in rural populations in Gabon: study protocol. SpringerPlus. 2014;3:388.

32. Adegnika AA, Verweij JJ, Agnandji ST, Chai SK, Breitling LP, Ramharter M, et al. Microscopic and sub-microscopic *Plasmodium falciparum* infection, but not inflammation caused by infection, is associated with low birth weight. Am J Trop Med Hyg. 2006;75:798–803.

33. Genser B, Cooper PJ, Yazdanbakhsh M, Barreto ML, Rodrigues LC. A guide to modern statistical analysis of immunological data. BMC Immunol. 2007;8:27.

34. Helmy A, Antoniades CA, Guilfoyle MR, Carpenter KLH, Hutchinson PJ. Principal component analysis of the cytokine and chemokine response to human traumatic brain injury. PLoS One. 2012;7:e39677.

35. Everts B, Adegnika AA, Kruize YCM, Smits HH, Kremsner PG, Yazdanbakhsh M. Functional impairment of human myeloid dendritic cells during *Schistosoma haematobium* infection. PLoS Negl Trop Dis. 2010;4:e667.

36. Meurs L, Labuda L, Amoah AS, Mbow M, Ngoa UA, Boakye DA, et al. Enhanced pro-inflammatory cytokine responses following Toll-like-receptor ligation in *Schistosoma haematobium*-infected schoolchildren from rural Gabon. PLoS One. 2011;6:e24393.

37. Van der Kleij D, van den Biggelaar AHJ, Kruize YCM, Retra K, Fillie Y, Schmitz M, et al. Responses to Toll-like receptor ligands in children living in areas where schistosome infections are endemic. J Infect Dis. 2004;189:1044–51.

38. Van Riet E, Everts B, Retra K, Phylipsen M, van Hellemond JJ, Tielens AGM, et al. Combined TLR2 and TLR4 ligation in the context of bacterial or helminth extracts in human monocyte derived dendritic cells: molecular correlates for Th1/Th2 polarization. BMC Immunol. 2009;10:9.

39. Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, et al. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J Immunol. 2003;171:4984–9.

40. Wammes LJ, Wiria AE, Toenhake CG, Hamid F, Liu KY, Suryani H, et al. Asymptomatic plasmodial infection is associated with increased tumor necrosis factor receptor II-expressing regulatory T

cells and suppressed type 2 immune responses. J Infect Dis. 2013;207:1590–9.

41. Turner JD, Meurs L, Dool P, Bourke CD, Mbow M, Dièye TN, et al. Schistosome infection is associated with enhanced whole-blood IL-10 secretion in response to cercarial excretory/secretory products. Parasite Immunol. 2013;35:147–56.

42. Smolen KK, Ruck CE, Fortuno 3rd ES, Ho K, Dimitriu P, Mohn WW, et al. Pattern recognition receptor-mediated cytokine response in infants across 4 continents. J Allergy Clin Immunol. 2014;133:818–26.

43. Labuda LA, de Jong SE, Meurs L, Amoah AS, Mbow M, Ateba-Ngoa U, et al. Differences in innate cytokine responses between European and African children. PLoS One. 2014;9:e95241.

44. Noone C, Parkinson M, Dowling DJ, Aldridge A, Kirwan P, Molloy SF, et al. Plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with *Plasmodium falciparum*. Malar J. 2013;12:5.

45. Diallo TO, Remoue F, Gaayeb L, Schacht A-M, Charrier N, De Clerck D, et al. Schistosomiasis coinfection in children influences acquired immune response against *Plasmodium falciparum* malaria antigens. PLoS One. 2010;5:e12764.

46. Netea MG, Quintin J, van der Meer JWM. Trained immunity: a memory for innate host defense. Cell Host Microbe. 2011;9:355–61

Chapter 7

The effect of helminth infections on cellular immune response of malaria infected subjects: A systematic review and meta-analysis of observational studies

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Manuscript in preparation

Abstract

Background

It has been postulated that helminths can influence the immune responses of malaria infected subjects. However, immuno-epidemiological studies report contradictory results. To summarize current available data and factors that may explain the differences observed we conducted a meta-analysis.

Methods

Articles were searched in 12 online databases. A random effect model was computed. Standardized mean difference and their 95% CIs were estimated using a "Restricted maximum likelihood estimator". The results were grouped to reflect the different arms of the cellular immune response. A moderator analysis was performed to understand the variations among the different studies.

Results

Of 1256 articles retrieved, 19 were eligible. Our main finding is that none of the immune parameters assessed in malaria infected subjects were influenced by helminth. Nonetheless, the results of the moderator analysis suggested that helminth species, past exposure to helminths, co-endemicity with multiple helminths and the type of immunological assays might explain this result.

Discussion and conclusion

This meta-analysis shows that a concurrent helminth infection does not influence the immune response of individuals co-infected with *P. falciparum*. However the moderator analysis also highlights the need for more standardized study designs and protocols to assess the interaction between helminths and malaria in immuno-epidemiological studies.

Introduction

Over the last decades, the question has arisen as to whether chronic helminth infections could have an impact on responses to a concurrent *Plasmodium spp.* infection. This question is important because helminths and malaria parasites share the same geographical distribution and frequently co-infect the same human host (1,2). From an immunological point of view, it has been demonstrated that helminths can exert potent modulatory effects on the immune system. A chronic helminth infection is usually marked by a Th2 polarized immune response (3) and by the induction of a regulatory network that can dampen the host immune response to the helminth itself (4–6), to other parasites (7,8) as well as to bystander antigens (9,10). A number of studies have examined the consequence of such immunological changes on malaria co-infection, but these have yielded variable results. In an attempt to determine the effect of helminths on immune responses during malaria infection in subjects living in areas endemic for both malaria and helminthiasis, we have performed a meta-analysis of the available observational studies. To our knowledge this is the first of its kind. Our objectives were firstly to specifically investigate the effect of helminths on response to infection with *Plasmodium spp.* and secondly to identify the factors that could explain the variability observed.

Methods

Literature search and selection of published articles

Literature search was carried out by an experienced librarian on the 13th of February 2014. Articles reporting on helminth and malaria co-infection in humans were searched in 12 different online databases. Databases included PubMed, MEDLINE, Embase, Web of Science, ScienceDirect as well as regional databases (See Appendix 1). The subject query was applied in all databases taking into account the terminological and technical differences between these databases. The query consisted of the combination of three subjects: helminths, malaria, and cellular immune response. Various synonyms and related terms for all subjects were used. Results were limited to human studies (see Appendix 2 for full details of the search queries). We did not

include any restriction on date of publication nor on language. Once identified, all articles were imported and stored into Reference Manager 12. Title and abstract of the identified articles were first screened for their relevance. Full texts of articles that passed this screening step were then consulted to check whether they were eligible for the meta-analysis. We considered eligible articles those that 1) reported results of a cross sectional, longitudinal or case control study, 2) were conducted in humans living in countries where helminth and malaria are endemic, 3) compared helminth and *Plasmodium spp.* co-infected subjects to subjects with single *Plasmodium spp.* infection 4) assessed the cellular immune response of the included participants. We excluded animal studies and case report studies in human. Studies were included regardless of their sample size, or whether or not they reported a randomization procedure for the selection of the participants.

Data extraction

Data were extracted from the eligible articles and saved in an electronic database. The type of data extracted were related to publication identification (author names, journal and year of publication) or to the study (objectives, study site, age group, type of helminths, Plasmodium species, type of immunological assays and the type of readouts for those assays as well as the statistical test used). In cases where multiple groups were assessed, we only extracted the data pertaining to the two groups of interest for our analysis. The mean and the standard deviation were extracted in order to calculate the effect size. When this information was not available in the text, they were calculated based on analysis of raw data provided by the authors or estimated from the available statistics. Authors were contacted when data were not available or when additional information was needed.

Statistical analysis

The statistical analysis was performed using R statistical software version 3.0.1 with his package metafor (version 1.9.2) and the integrated development environment Rstudio version 0.97.551. We used a random effect model for our meta-analysis since we assumed a within study random error as well as variation in effect size from one

study to the next that could lead to heterogeneity among the measured true effect.

Standardized mean difference (SMD) and their 95% CIs were estimated using a "Restricted maximum likelihood estimator" (REML). For a more comprehensible and systematic approach results reported in the selected articles were grouped in categories reflecting the Th1, Th2 or Th17 arm of the cellular immune response. Additional groups included results reporting on regulatory cytokines and regulatory T cells. Authors usually report results of more than one cytokine, for example TNF and IFN-γ for the Th1 cytokine group. Therefore our data were not considered independent and a multiple treatment meta-analysis was performed to take into account the dependency of the data. The unit of analysis was the article identification number.

A sensitivity analysis was conducted to assess the influence of moderators on the robustness of the results. The following moderator variables were considered 1) the type of helminth assessed (Schistosoma only, intestinal helminths only, filaria only or Schistosoma plus intestinal helminths), 2) the source of the cytokines (serum or plasma, intracellular or supernatants of stimulated cells) and 3) the type of stimuli used for stimulated cells (iRBCs, Malaria antigens, MSP1-19, LPS, LPS + Zymozan, PHA or PMA + ionomycin).

Results

Description of the selected articles

Figure 1 describes the selection process of the articles included in the meta-analysis. A description of the 19 eligible articles is given in table 1. The majority of studies (n = 17) were conducted in Africa (Nigeria:2 (22,25), Senegal:3 (26–28), Mali:7(11,13–15,19,21,23), Ghana:3 (8,18,20), Kenya: 2(16,17)) and two in Asia (India:1 (12), Indonesia:1(24)). Twelve studies were cross-sectional (8,14,16–20,22,24,25,27,28), 3 used case-control design (12,15,21), 1 was longitudinal (26) and 3 studies from the same authors combined a longitudinal and a case control design (11,13,23). The age range varied but majority of the studies (17 in total) included school aged children, whereas 1 focused on children aged from 3 to 6 (22) and two were

conducted in adults (12,28). When added together the total sample size was 2501 subjects (table 1).

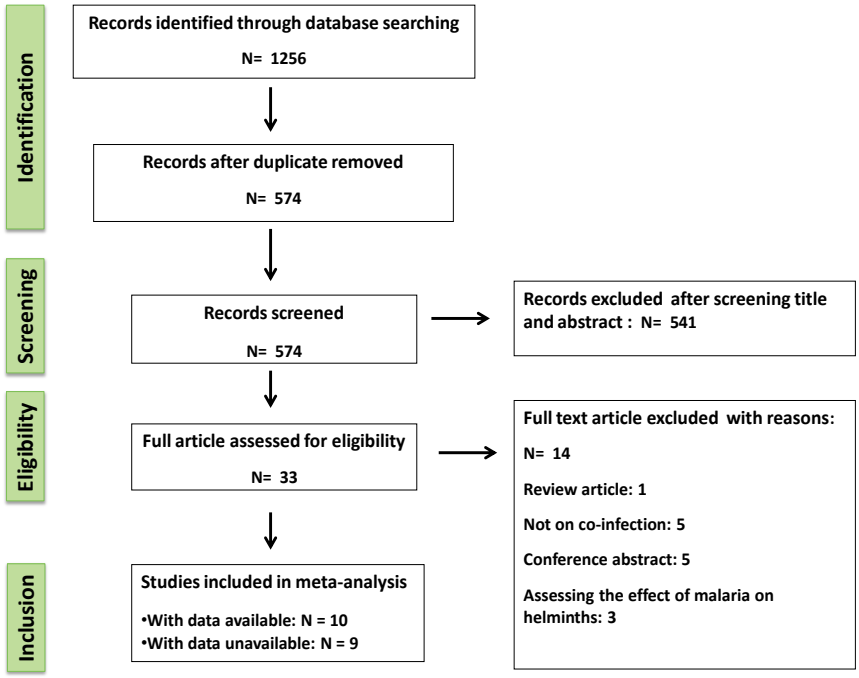

Figure 1: Flow chart showing the selection process of the articles included in the meta-analysis

Th1 and pro-inflammatory response

A total of 18 articles assessed the effect of helminths on the Th1/pro-inflammatory immune response of malaria co-infected subjects (8,12–28). Eight of the articles were not included in the meta-analysis because no raw data were available (12,15,17–19,24,26,28). From the 10 articles that were used, the Th1/pro-inflammatory responses were characterized based on the levels of IFN-γ (8,13,14,16,20–22,25,27), TNF (8,13,14,16,20–22,25), IL-12 (27), IL-12p40 (16), IL12-p70 (13,21), IL-2 (11,13,22), IL-8 (13,25), IL-1b (13), sTNFr (16). These analytes were measured either in the plasma/serum of the study

participants (13,16,22,25) or in supernatants obtained after stimulation of whole blood or PBMC (8,14,18,21,27). Intracellular cytokines in T cells were also taken along (14). The result of our meta-analysis is displayed in figure 2. Results indicate variation on the standardized mean difference of the cytokine of interest. For example in the case of TNF, 2 out of the 12 effect sizes reported showed a decrease in its level in helminth/malaria co-infected subjects in comparison with subjects with malaria only. In one study an increase of the effect size was observed in co-infected subjects whereas in 8 cases no significant difference was observed between malaria single infected and co-infected subjects. The pooled standardized mean difference was not significantly different between co-infected and single *P. falciparum* infected subjects (TNF SMD =-0.44 (95%CI [-1.33, 0.45], p = 0.33). This implies that helminth infections are not associated with a significant impairment of TNF response in malaria infected subjects although a trend toward a decrease was seen. IFN-γ another important Th1 cytokine was assessed in 9 studies that reported 16 different results. As shown in Figure 2, 2/16 results were in line with an increase of IFN-γ levels in co-infected subjects and 1/16 showed a decrease of this cytokines in the coinfected group by comparison to *P. falciparum* single infected subjects. In 13/16 studies no significant effect of helminths on IFN-γ response was seen in subjects with malaria. The pooled estimate of the effect size showed no overall effect of helminths on IFN-γ response (IFN-γ SMD = 0, 95%CI [-1.36, 1.36], p = 1). Similarly no effect of helminths was observed on the levels of IL-2, IL-8 or IL-12. When data of all the Th1/Pro-inflammatory cytokines were pooled, we failed to find a significant difference between helminth and malaria co-infected subjects in comparison to subjects with single *P. falciparum* infection (Overall SMD =0.02, 95% CI[-0.46, 0.49], p = 0.9).

Table 1: Characteristics of the studies included in the meta-analysis

N°	Authors (year of publication)	Study design	Country	Age range (in years)	Sample size *n*	Helminth species	Plasmodium species	Clinical malaria or asymptomatic carriage of *P. falciparum*	Immunological assay performed	Stimuli used if cells were stimulated	Read out
1	Noone et al. (2013)	Cross-sectionnal	Nigeria	3 to 6	231	*Ascaris Lumbricoides*	*P. falciparum*	Clinical malaria	Plasma cytokine	NA	Plasma cytokines
2	Courtin et al. (2011)	Longitudinal (but immunological assays done at one time point)	Senegal	6 to 19	305	*S. haematobium*	*P. falciparum*	Both	Plasma cytokine	NA	Plasma cytokines
3	Diallo et al. (2010)	Cross-sectional	Senegal	7 to 15	79	*S. haematobium*	*P. falciparum*	Asymptomatic carriage	Whole blood culture	MSP1 antigens and *P. falciparum* schizont lysate	Cytokines measured in the cell culture supernatant
4	Dolo et al. (2012)	Case control	Mali	4 to 20	24	*Wuchereria brancofti* and *Mansonella perstans*	*P. falciparum*	Clinical malaria	Plasma cytokine	NA	Plasma cytokine
5	Hartgers et al. (2009)	Cross-sectional	Ghana	6 to 12	117	*S. haematobium* and *intestinal helminths*	*P. falciparum*	Asymptomatic carriage	Whole blood culture	*P. falciparum* iRBCs	Cytokines measured in the cell culture supernatant

Abbreviations used: iRBC: infected Red Blood cells, PBMC: Peripheral Blood Mononuclear Cells, SEA: Soluble Eggs Antigens, SWA: Soluble Worm Antigen, SWAP: Soluble Worm Antigen Protein, AMA1: Apical membrane antigen 1, MSP1: Merozoite Surface Protein 1, PHA: Phytohaemagglutinin, NA: Not Applicable

Table 1 (*contd*): Characteristics of the studies included in the meta-analysis

N°	Authors (year of publicatio)	Study design	Country	Age range (in year)	Sample size *n*	Helminth species	Plasmodium species	Clinical malaria or asymptomatic carriage of *P. falciparum*	Immunological assay performed	Stimuli used if cells were stimulated	Read out
6	Lyke et al. (2006)	Nested case control	Mali	4 to 14	505	*S. haematobium*	*P. falciparum*	Clinical malaria	Serum cytokine	NA	Serum cytokine
7	Metenou et al. (2009)	Case-control	Mali	11 to 20	38	*Wuchereria brancofti* and *Mansonella perstans*	*P. falciparum*	No current plasmodium infection	Whole blood culture	iRBCs	Cytokines measured in cell culture supernatant
8	Metenou et al. (2011)	Cross-sectional	Mali	11 to 18	28	*Wuchereria brancofti* and *Mansonella perstans*	*P. falciparum*	Asymptomatic carriage	Whole blood culture	iRBCs	Intracellular cytokines
9	Muok et al. (2009)	Cross-sectional	Kenya	8 to 10	153	*Schistosoma mansoni*	*P. falciparum*	Not clear	Whole blood collected and stained withouth stimulation	NA	T cells characterized by surface markers
10	Nmorsi (2009)	Cross-sectional	Nigeria	1 to 15	160	*S. haematobium*	*P. falciparum*	Clinical malaria	Plasma cytokines	NA	Plasma cytokines
11	Wilson et al. (2009)	Cross-sectional	Kenya	4 to 17	228	*S. mansoni*	*P. falciparum*	Asymptomatic carriage	Plasma cytokines	NA	Plasma cytokines

Abbreviations used: iRBC: infected Red Blood cells, PBMC: Peripheral Blood Mononuclear Cells, SEA: Soluble Eggs Antigens, SWA: Soluble Worm Antigen, SWAP: Soluble Worm Antigen Protein, AMA1: Apical membrane antigen 1, MSP1: Merozoite Surface Protein 1, PHA: Phytohaemagglutinin, NA: Not Applicable

Table 1 (contd): Characteristics of the studies included in the meta-analysis

N°	Authors (year of publication)	Study design	Country	Age range (in years)	Sample size *n*	Helminth species	Plasmodium species	Clinical malaria or asymptomatic carriage of *P. falciparum*	Immunological assay performed	Stimuli used if cells were stimulated	Read out
12	Lyke et al. (2012)	Case control/longitudinal	Mali	4 to 14	38	*S. haematobium*	*P. falciparum*	Clinical malaria	Surface staining of PBMC	NA	T cells characterized by surface markers
13	Metenou et al. (2012)	Cross-sectional	Mali	11 to 18	35	*Wuchereria brancofti* and *Mansonella perstans*	*P. falciparum*	No current plasmodium infection	1.Whole blood culture 2.Gene expression 3.cDNA synthesis 4.RT-PCR	iRBCs	1.RNA 2.mDCs, pDCs
14	Panda et al. (2013)	Case control	India	Not determined but seems to be adult	234	Filaria (genus and species not specified in the text)	*P. falciparum*	Clinical malaria	1.Plasma cytokine 2.Whole blood	NA	1. Plasma cytokine 2.Regulatory T cells characterized by surface markers
15	Wammes et al. (2010)	Cross-sectionnal	Indonesia	School age children	20	Geo helminths	*P. falciparum*	No current plasmodium infection	1.Cell isolation, depletion and phenotyping 2.Proliferation assay	iRBCs	1.T cells characterization2.T cells proliferation 3.Cytokines in cell culture supernatatant

Abbreviations used: iRBC: infected Red Blood cells, PBMC: Peripheral Blood Mononuclear Cells, SEA: Soluble Eggs Antigens, SWA: Soluble Worm Antigen, SWAP: Soluble Worm Antigen Protein, AMA1: Apical membrane antigen 1, MSP1: Merozoite Surface Protein 1, PHA: Phytohaemagglutinin, NA: Not Applicable

Table 1 (contd): Characteristics of the studies included in the meta-analysis

N°	Authors (year of publication)	Study design	Country	Age range (in years)	Sample size *n*	Helminth species	Plasmodium species	Clinical malaria or asymptomatic carriage of *P. falciparum*	Immunological assay performed	Stimuli used if cells were stimulated	Read out
16	Wilson et al. (2009)	Cross-sectionnal	Kenya	4 to 17	79	*S. mansoni*	*P. falciparum*	Asymptomatic carriage	Whole blood culture	SEA, SWA and PHA	Cytokines in cell culture supernatatant
17	Hartgers et al. (2008)	Cross-sectional	Ghana	6 to 13	16	*S. haematobium and intestinal helminths*	*P. falciparum*	Asymptomatic carriage	Whole blood culture	iRBCs	Cytokines measured in the cell culture supernatant
18	Lyke et al. (2012)	Case control/ longitudinal	Mali	4 to 14	84	*S. haematobium*	*P. falciparum*	Clinical malaria	1.PBMC culture.2.Memory B cells assays 3.ELISA.4.Flow cytometry staining.5.EIISPOT	SEA, SWA, AMA1, MSP1	1.Antibodies, 2.Memory B cells response to malaria or schistosoma antigens
19	Diallo et al. (2004)	Cross-sectional	Sénégal	Children from 7 to 15 and adult more than 30 years old	Children: 79 Adult: 48	*S. haematobium*	*P. falciparum*	Asymptmatic carriage	Plasma cytokines	NA	Plasma cytokines

Abbreviations used: iRBC: infected Red Blood cells, PBMC: Peripheral Blood Mononuclear Cells, SEA: Soluble Eggs Antigens, SWA: Soluble Worm Antigen, SWAP: Soluble Worm Antigen Protein, AMA1: Apical membrane antigen 1, MSP1: Merozoite Surface Protein 1, PHA: Phytohaemagglutinin, NA: Not Applicable

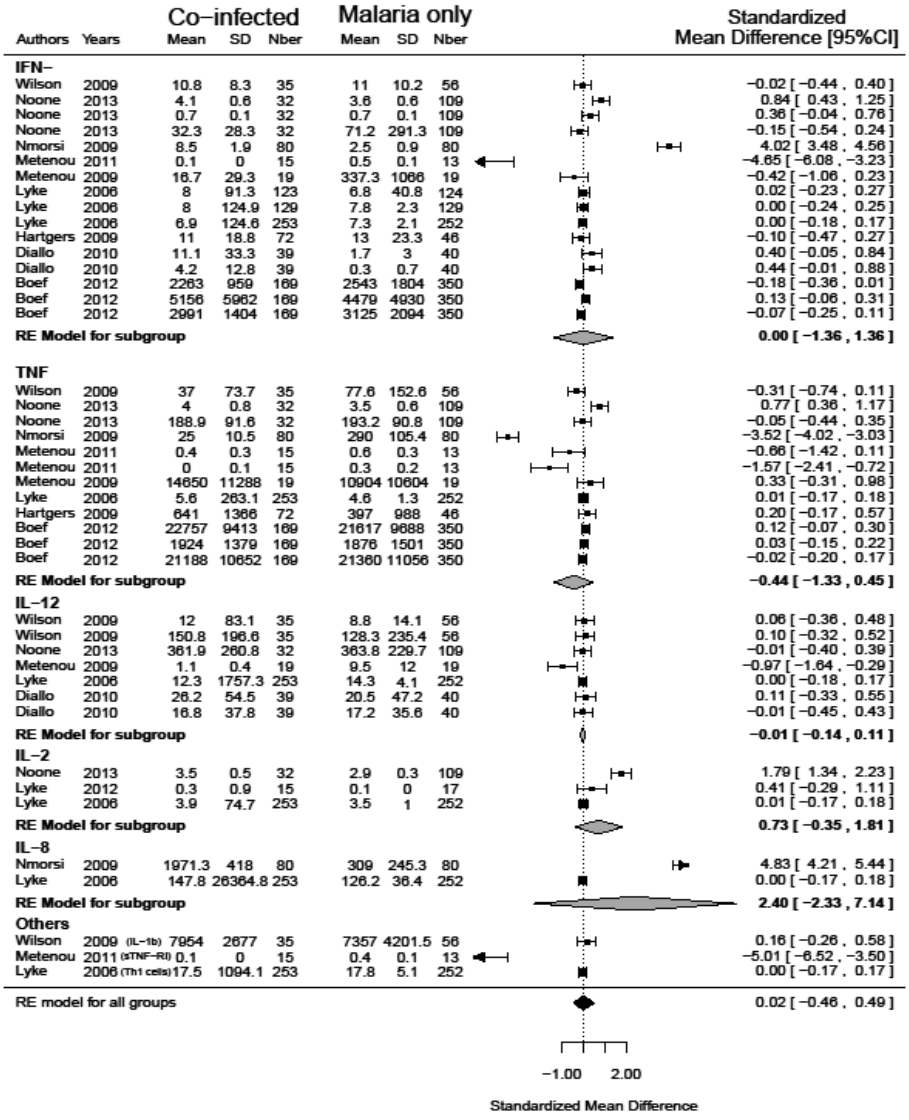

Figure 2: Forest plot of meta-analysis investigating the effect of helminth co-infection on the Th1/pro-inflammatory cytokines of *P. falciparum* infected subjects. The standardized mean difference (SMD) for each experiment is shown as a black square with 95% confidence intervals (CIs). The blue diamond represents the pooled estimate of the SMD for subgroup while the pooled estimate for all groups is shown at the bottom of the figure. Positive SMD indicates an increase of the Th1/pro-inflammatory cytokines in helminth and *P. falciparum* co-infected subjects by comparison to subjects with *P. falciparum* infection only.

Th2 cytokines

From the articles reporting on Th2 immune response that were eligible for the meta-analysis, 8 reported results on IL-4, IL-5, IL-13 cytokines that are known to be part of the Th2 response(8,13,14,16,20,22,25,27). In addition Metenou *et al.* assessed the Th2 immune response by characterizing multifunctional T cells that express IL-4, IL-5 and/or IL-10 (14). As depicted in figure 3 not much variation was observed in the reported effect of helminths on malaria regarding the Th2 cytokine responses assessed. Regardless of the cytokine, no difference was observed between the helminth and malaria co-infected compared to those subjects with *P. falciparum* only. The only exception to this observation was an increase of IL-4 and IL-5 in co-infected subjects reported by Nmorsi *et al.*(25) and an increase of IL-13 as indicated by the data from by Boef *et al.* (20).The overall pooled effect size did not reveal any difference between the two groups compared (Overall SMD = 0.24, 95% CI [-0.22, 0.69], p = 0.3).

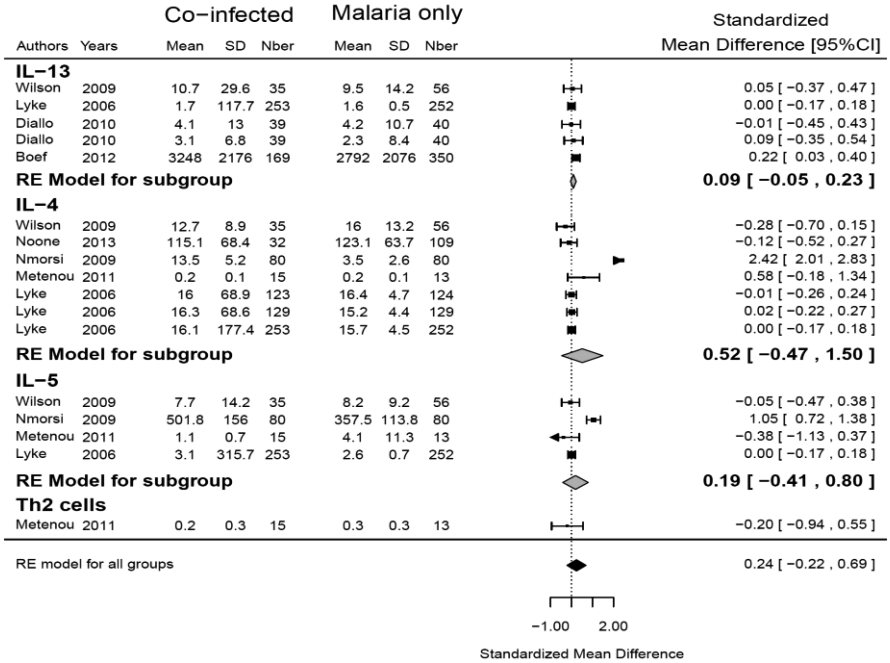

Figure 3: Forest plot of meta-analysis investigating the effect of helminth co-infection on the Th2 cytokines cytokines of *P. falciparum* infected subjects. The standardized mean difference (SMD) for each experiment is shown as a black square with 95% confidence intervals (CIs). The blue diamond represents the pooled estimate of the SMD for subgroup while the pooled estimate for all groups is shown at the bottom of the figure. Positive SMD indicates an increase of the Th2 cytokines in helminth and *P. falciparum* co-infected subjects by comparison to subjects with *P. falciparum* infection only.

Regulatory cytokines

IL-10 and TGF-β were the two regulatory cytokines studied (figure 4). The effect sizes of these cytokines were reported in 10 different articles that had generated 20 different results (8,13,14,16,18,20–22,25,27). A total of 16 different results obtained from 9 articles (8,13,14,16,20–22,25,27) reported on IL-10 which was found to be significantly higher in co-infected subjects for 4/16 studies (8,14,21,27). Conversely 1/16 showed a significant decrease of this cytokine in the co-infected group by comparison to subjects with *P. falciparum* only (13). Overall the meta-analysis revealed no significant effect of helminths on the IL-10 levels of malaria infected subjects despite a tendency for an increase in co-infected group (IL-10 SMD = 0.22, 95% CI [-0.12, 0.55], p =0.21). On the other hand data on the TGF-β were available from 3 articles which reported 4 results (16,22,27). Overall no differences were observed between co-infected and the malaria only group (TGF-β SMD= -0.12, 95% CI [-0.33, 0.09], p = 0.26). Finally the overall pooled estimate was not significantly different between the two groups that we compared (Overall SMD = 0.18, 95% CI [-0.14, 0.49], p =0.26).

Th17 cytokines

Data on the Th17 cytokines were available from 4 articles (14,20–22). They reported 7 different results that were included in the meta-analysis shown in Figure 5. A significant decrease of IL-17 was observed in 2 of the results in the helminth and malaria coinfected group (14,21). The remaining five available results did not show a significant effect of helminths on Th-17 response to *P. falciparum* infected subjects. Finally the pooled effect size did not differ significantly between the groups (Overall SMD =-0.17, 95% CI [-0.59, 0.24], p = 0.41)

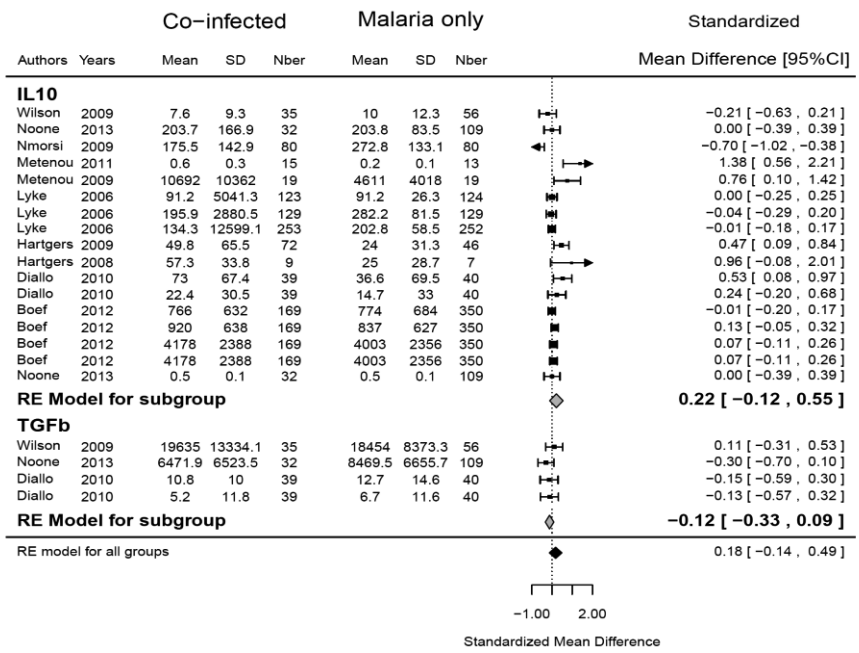

Figure 4: Forest plot of meta-analysis investigating the effect of helminth co-infection on the regulatory cytokines of *P. falciparum* infected subjects. The standardized mean difference (SMD) for each experiment is shown as a black square with 95% confidence intervals (CIs). The blue diamond represents the pooled estimate of the SMD for subgroup while the pooled estimate for all groups is shown at the bottom of the figure. Positive SMD indicates an increase of the regulatory cytokines in helminth and *P. falciparum* co-infected subjects by comparison to subjects with *P. falciparum* infection only.

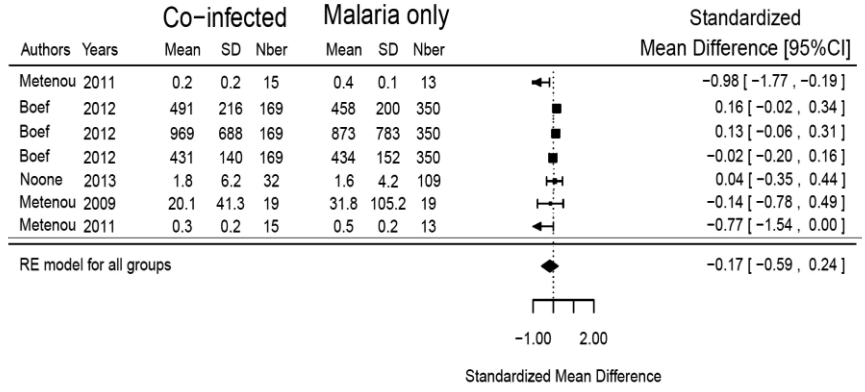

Figure 5: Forest plot of meta-analysis investigating the effect of helminth co-infection on the Th17 cytokines of *P. falciparum* infected subjects. The standardized mean difference (SMD) for each experiment is shown as a black square with 95% confidence intervals (CIs). The blue diamond represents the pooled estimate of the SMD for subgroup while the pooled estimate for all groups is shown at the bottom of the figure. Positive SMD indicates an increase of the Th17

cytokines in helminth and *P. falciparum* co-infected subjects by comparison to subjects with *P. falciparum* infection only.

Regulatory T cells

Data on the Regulatory T cells (Treg) were available from two different studies (11,14). They reported opposite effects. In one of these studies an increase in the frequency of Treg was reported (SMD= 0.86, 95%CI [0.09. 1.64]) (11) whereas in the other a lower frequency of Treg was observed (14) in co-infected subjects (SMD=-1.02, 95%CI [-1.69, -0.34]). The overall pooled effect was not significantly different between the groups (Overall SMD = -0.09, 95%CI [-1.93, 1.76], p = 0.9).

Publication bias

The presence of publication bias was assessed for the pooled effect size of the Th1, Th2 and the regulatory cytokine responses by scrutinizing their respective funnel plot. As shown in figure 6 funnel plots of the Th1 and regulatory cytokine responses show a roughly symmetrical structure indicating the absence of publication bias. Funnel plot for Th2 showed a slight asymmetry. Presence of publication bias was not assessed for the Th17 response nor for the regulatory T cells due to the small number of studies (less than 10).

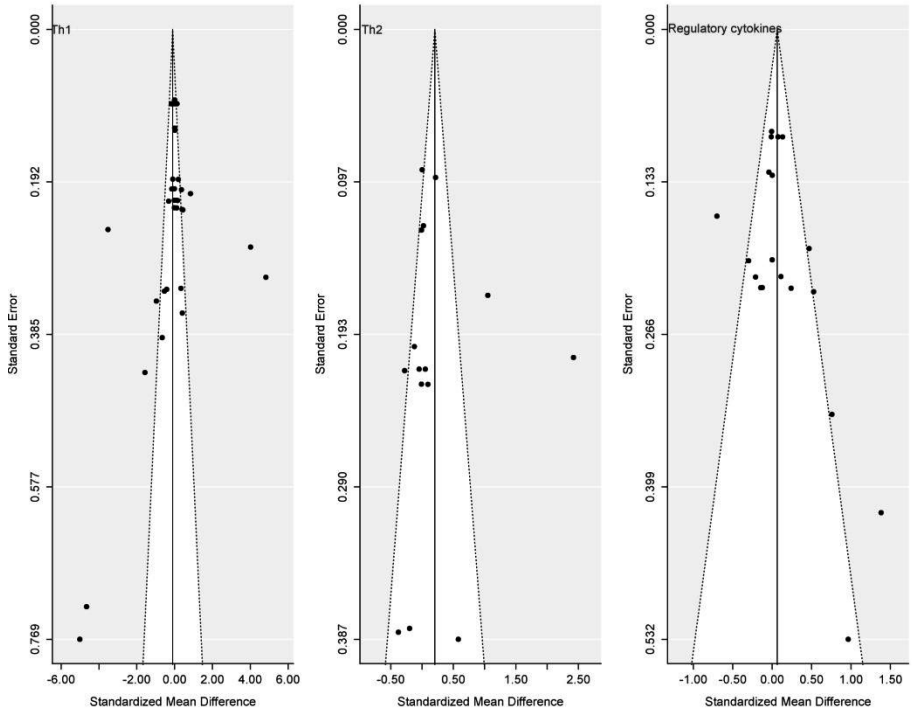

Figure 6: Funnel plot analysis to detect publication bias. The left panel shows funnel plot performed on the results published for the Th1/Pro-inflammatory cytokines whereas the middle and the right panels show funnel plots for the Th2 and the Regulatory cytokines. Each point represents a single result.

Moderator analysis

We conducted a moderator analysis to determine to what extent differences in methodologies or the characteristics of the populations assessed could have influenced the outcome of our meta-analysis. The moderator analysis was performed on the pooled estimate of the Th1, Th2, Th17 and regulatory cytokines effect size. The moderator analysis was not conducted on the effect size of the Regulatory T cells since only two results were available. As shown in Table 2 we observed that of the helminths studied filaria infection was associated with a significant decrease of the Th1 and the Th17 response in subjects with malaria (respectively Th1 SMD = -1.1, 95%CI [-2, -0.2], p =0.01 and Th17 SMD = -0.5, 95%CI[-1,-0.1], p = 0.008) along with a significant increase of the regulatory cytokines in the same group (Regulatory cytokines SMD = 1.02, 95%CI [0.4, 1.7], p = 0.001) suggesting a filaria

specific effect. It was noted that studies on filarial infection utilised an IgG diagnostic test that could also exclude past exposure to filarial infection. An increase of the regulatory cytokines was also observed when subjects with malaria were co-infected with both schistosoma and intestinal helminths (Regulatory cytokines SMD = 0.6, 95%CI [0.02, 1.1], p=0.04). This might indicate that not only the species of helminths but also the number of helminths species infecting the human host might influence the effect on the host immune system. When considering the second moderator variable (type of cytokines measurement) we noted that the pooled estimate of the Th17 response was higher in the co-infected group when this cytokine was measured intracellularly (Th17 SMD = -0.9, 95%CI[-1.4, -0.3], p = 0.002). Finally we observed that the use of PHA as a stimulus resulted in a significantly higher effect size of the Th2 response in the co-infected group (Th2 SMD = 0.2, 95%CI [0.09, 0.4], p = 0.008) whereas the use of iRBCs led to a significant increase in the regulatory cytokines in the same group (Regulatory cytokines SMD = 0.6, 95%CI [0.2, 1.01], p = 0.002).

Table 2: Result of the moderator analysis

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Moderators variables	Levels	Th1			Th2			Regulatory cytokines			Th17		
		N° of studies	SMD [95%CI]	p	N° of studies	SMD [95%CI]	p	N° of studies	SMD [95%CI]	p	N° of studies	SMD [95%CI]	p
Type of helminths	Intestinal helminths	13	0.2 [-0.6, 1.1]	0.6	3	0.05 [-0.8, 0.9]	0.9	6	-0.01 [-0.4, 0.3]	1	4	0.1 [-0.01, 0.2]	0.1
	Filaria*	7	1.1 [-2, -0.2]	0.01	4	0.02 [-0.9, 1]	1	3	1.02 [0.4, 1.7]	0.001	3	-0.5 [-1, -0.1]	0.008
	Schistosoma	21	0.3 [-0.2, 0.9]	0.2	16	0.4 [-0.2, 1.02]	0.2	10	-0.14 [-0.4, 0.14]	0.3	-	-	-
	Schistosoma and intestinal helminths	2	0.04 [-1.2, 1.3]	0.9	1	0.3 [-0.9, 1.6]	0.6	2	0.6 [0.02, 1.1]	0.04	-	-	-
Type of cytokine measurement	Plasma and serum Cytokines	19	-0.4 [-1.2, 0.5]	0.4	15	0.4 [-0.2, 0.9]	0.2	9	-0.1 [-0.5, 0.2]	0.5	1	0.04 [-0.4, 0.4]	0.8
	Cytokines measured after cells stimulation	15	-0.01 [-1.03, 1.01]	1	6	0.2 [-0.4, 0.8]	0.5	10	0.4 [-0.01, 0.7]	0.06	4	0.08 [-0.02, 0.2]	0.1
	Intracellular cytokines	9	0.5 [-0.3, 1.4]	0.2	3	-0.005 [-1.2, 1.2]	1	2	0.3 [-0.2, 0.8]	0.3	2	-0.9 [-1.4, -0.3]	0.002

SMD: Standardized mean difference

Table 2 *(contd)*: Result of the moderator analysis

Moderators variables	Levels	Th1			Th2			Regulatory cytokines			Th17		
		N° of studies	SMD [95%CI]	p	N° of studies	SMD [95%CI]	p	N° of studies	SMD [95%CI]	p	N° of studies	SMD [95%CI]	p
Type of stimuli used in case cytokines were measured after cells stimulation	iRBCs	11	-0.4 [-1.4, 0.4]	0.3	6	0.1 [-0.1, 0.3]	0.3	6	0.6 [0.2, 1.01]	0.002	3	-0.5 [-1.2, 0.2]	0.1
	Malaria antigens	1	0.4 [-1.6, 2.4]	0.7	-	-	-	-	-	-	-	-	-
	MSP1-19	2	-0.5 [-1.5, 0.5]	0.3	1	0.09 [-0.3, 0.5]	0.7	2	0.4 [-0.9, 0.1]	0.1	-	-	-
	LPS	2	-0.04 [-1.9, 1.8]	1	-	-	-	1	0.07 [-0.6, 0.7]	0.8	1	0.2 [-0.7, 1]	0.7
	LPS+Zymozan	2	-0.03 [-1.9, 1.8]	1	-	-	-	1	-0.01 [-0.7, 0.7]	1	1	-0.02 [-0.9, 0.8]	1
	PHA	2	0.08 [-1.8, 2]	0.9	2	0.2 [0.09, 0.4]	0.008	1	0.13 [-0.6, 0.8]	0.7	1	0.1 [-0.7, 1]	0.8
	PMA/Ionomycin	4	0.9 [-1, 3]	0.3		-	-	1	0 [-0.8, 0.8]	1	-	-	-

SMD: Standardized mean difference

Discussion

The Th1 response has been shown to be important for control of *Plasmodium spp*. infection. Experimental animal studies have indicated that mice that lack the ability to produce IFN-γ were unable to control the malaria parasites and died (29). In humans, Th1 type and pro inflammatory responses are also important for *P. falciparum* specific immunity since increased levels of IFN-γ and TNF have repeatedly been observed in malaria infected subjects and have been linked with protection from infection (30,31). In Gabon, a longitudinal study showed that individuals with an IFN-γ response to *P. falciparum* liver stage antigens had a significantly delayed time to re-infection and a low rate of re-infection compared to their non–responders counterpart (32).

Helminths known as strong inducers of the Th2 response have been shown to increase (15,22,25,27,28), decrease (13,14,21,25) or have no effect (8,12–16,19,20,22,27,28) on Th1 response in *P. falciparum* infected individuals. These studies have been included in the current meta-analysis and when pooled together yielded an effect size that suggests no significant impact of helminths on Th1 response to malaria.

In humans, IL-4 (31,33) and IL-13 (31) have been reported to be increased in individuals with uncomplicated malaria and might be further expanded in helminth co-infected subjects. Here out of the 23 results included in the meta-analysis only two showed an increase of IL-4 and IL-5 respectively in the co-infected groups. Furthermore the pooled effect did not yield any significant difference between the two groups despite a tendency for an increase in subjects co-infected with helminth and malaria. Similarly the results of the regulatory response and Th17 which has been shown to be expanded during *P. falciparum* infection did not show any change as a result of helminth co-infection.

People are usually exposed to helminth antigens already in utero (34), they get infected early in life (35) and most of the time they are infected with more than one helminth species (36). In this context it would be difficult to identify individuals that are truly free of helminths not only for current but also for past infections. It is most likely that the helminth free subjects included in the majority of the studies were

infected with more than one type of helminths, or had been exposed to helminths antigens prior to their participation in the studies. This is of particular interest for the interpretation of our results since; i) past exposure to helminth antigens could imprint the host immune system in lasting manner (37) and ii) host immune system could be modulated by antigens from different helminths species (38). One major limitation of the studies included in this meta-analysis is that most of them were cross sectional in design thus could not provide information on past exposure to helminths. Moreover as already alluded to, the studies mainly focused on one helminth species and therefore were not able to assess the confounding effect of other helminth infections.

This is supported by the moderator analysis we conducted to assess whether the effect of helminths on the cytokine response of *P. falciparum* infected subjects was dependent on the species of helminths assessed. Of the different helminth species examined in the studies included we noted that malaria co-infection with filaria in an area where no other helminths were endemic was associated with a significant decrease in the Th1/Pro-inflammatory as well as the Th17 response and with an increase of regulatory cytokines. However only two studies assessed the effect of filarial parasites on the immune response of malaria infected subjects and these were both reported by the same authors. Furthermore microscopic examination was used to determine current filarial infection whereas past infection with/exposure to filaria was ascertained by the detection of IgG to filarial antigens (14,21). With such a methodology it was possible to identify subjects that were truly free of filarial infection and therefore possible to show an effect of this helminth on the Th1 response of *P. falciparum* infected subjects.

The moderator analysis we performed was of particular importance in determining the factors that could influence the result of our meta-analysis. In addition to the type of helminths the details of the immunological assays used were important. For example the Th2 response was more pronounced in individuals coinfected with helminths and *P. falciparum* if PHA was used as stimulus. Similarly regulatory cytokine were more pronounced in subjects from the co-infected group if their cells were cultured with iRBCs rather than any other stimulus. Taken together these observations might indicate that

heterogeneity is mainly created by the various study designs and methodologies used. However this should not be seen as an obstacle for conducting a meta-analysis since the first aim of such an approach is to summarize studies that have inherent differences. Furthermore, it is possible, to use a random effect model (as we did here), to take into account the observed heterogeneity. Limitations of this meta-analysis are related to the fact that in the majority of the articles included, the selection of participants was not randomized, studies were cross sectional or sample sizes were small. These situations are well known and well described for meta-analyses conducted on observational studies that are by definition less strict than meta-analysis of randomized controlled trials in design (39–41). However due to the importance of such an analysis for summarizing results and informing researchers, meta-analysis is preferred to narrative review (40). It could be misleading to extend our results to all the components of the immune system. Indeed with the advances in technologies, the immune system appears more and more complex and this meta-analysis has only considered a fraction of this complexity. For example, due to the paucity of data, we were not able to assess whether the B cells or antibody responses of malaria infected subjects were affected by a concurrent helminth infection. Similarly neither cells involved in innate immunity nor the distribution of the different T cells subsets was assessed.

In conclusion this meta-analysis summarizes the results of immuno-epidemiological studies that assessed the effect of helminths on the cellular immune response of *P. falciparum* infected subjects. Our results indicate that current helminth infections do not affect *P. falciparum* associated immune response. However it is not clear whether this is due to the fact that individuals considered as free of helminths could have been exposed earlier in their life or could have harboured other helminth species also capable of modulating their immune response. Further studies will be needed to address this. Moreover this meta-analysis also highlights the need for more standardized study design and methodologies to assess the effect of helminths on *P. falciparum* specific immune response.

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References

1. Brooker S, Akhwale W, Pullan R, Estambale B, Clarke SE, Snow RW, et al. Epidemiology of plasmodium-helminth co-infection in Africa: populations at risk, potential impact on anemia, and prospects for combining control. Am J Trop Med Hyg. 2007 Dec;77(6 Suppl):88–98.

2. Mwangi TW, Bethony J, Brooker S. Malaria and helminth interactions in humans: an epidemiological viewpoint. Ann Trop Med Parasitol. 2006 Oct;100(7):551–70.

3. van Riet E, Hartgers FC, Yazdanbakhsh M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. Immunobiology. 2007;212(6):475–90.

4. Taylor JJ, Mohrs M, Pearce EJ. Regulatory T Cell Responses Develop in Parallel to Th Responses and Control the Magnitude and Phenotype of the Th Effector Populatio. J Immunol. 2006 May 15;176(10):5839–47.

5. Sawant DV, Gravano DM, Vogel P, Giacomin P, Artis D, Vignali DAA. Regulatory T cells limit induction of protective immunity and promote immune pathology following intestinal helminth infection. J Immunol Baltim Md 1950. 2014 Mar 15;192(6):2904–12.

6. Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, Maizels RM. Removal of Regulatory T Cell Activity Reverses Hyporesponsiveness and Leads to Filarial Parasite Clearance In Vivo. J Immunol. 2005 Apr 15;174(8):4924–33.

7. Wammes LJ, Hamid F, Wiria AE, de Gier B, Sartono E, Maizels RM, et al. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. Eur J Immunol. 2010 Feb;40(2):437–42.

8. Hartgers FC, Obeng BB, Kruize YCM, Dijkhuis A, McCall M, Sauerwein RW, et al. Responses to malarial antigens are altered in helminth-infected children. J Infect Dis. 2009 May 15;199(10):1528–35.

9. Esen M, Kremsner PG, Schleucher R, Gässler M, Imoukhuede EB, Imbault N, et al. Safety and immunogenicity of GMZ2 - a MSP3-GLURP fusion protein malaria vaccine candidate. Vaccine. 2009 Nov 16;27(49):6862–8.

10. Cooper PJ, Espinel I, Paredes W, Guderian RH, Nutman TB. Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocerciasis: a possible role for interleukin-10. J Infect Dis. 1998 Oct;178(4):1133–8.

11. Nmorsi O, Isaac C, Ukwandu N, Ekundayo A, Ekozien M. Schistosoma haematobium and Plasmodium falciparum co-infection with protection against Plasmodium falciparum malaria in Nigerian children. Asian Pacific journal of tropical medicine. :16–20.

12. Noone C, Parkinson M, Dowling DJ, Aldridge A, Kirwan P, Molloy SF, et al. Plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with Plasmodium falciparum. Malar J. 2013;12:5.

13. Diallo TO, Remoue F, Schacht AM, Charrier N, Dompnier J-P, Pillet S, et al. Schistosomiasis co-infection in humans influences inflammatory markers in uncomplicated Plasmodium falciparum malaria. Parasite Immunol. 2004 Sep;26(8-9):365–9.

14. Diallo TO, Remoue F, Gaayeb L, Schacht A-M, Charrier N, De Clerck D, et al. Schistosomiasis coinfection in children influences acquired immune response against Plasmodium falciparum malaria antigens. PloS One. 2010;5(9):e12764.

15. Courtin D, Djilali-Saïah A, Milet J, Soulard V, Gaye O, Migot-Nabias F, et al. Schistosoma haematobium infection affects Plasmodium falciparum-specific IgG responses associated with protection against malaria. Parasite Immunol. 2011 Feb;33(2):124–31.

16. Lyke KE, Wang A, Dabo A, Arama C, Daou M, Diarra I, et al. Antigen-specific B memory cell responses to Plasmodium falciparum malaria antigens and Schistosoma haematobium antigens in co-infected Malian children. PloS One. 2012;7(6):e37868.

17. Lyke KE, Dabo A, Sangare L, Arama C, Daou M, Diarra I, et al. Effects of concomitant Schistosoma haematobium infection on the serum cytokine levels elicited by acute Plasmodium falciparum malaria infection in Malian children. Infect Immun. 2006 Oct;74(10):5718–24.

18. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly YI, Diallo AA, et al. Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol Baltim Md 1950. 2011 Apr 15;186(8):4725–33.

19. Metenou S, Kovacs M, Dembele B, Coulibaly YI, Klion AD, Nutman TB. Interferon regulatory factor modulation underlies the bystander suppression of malaria antigen-driven IL-12 and IFN-γ in filaria-malaria co-infection. Eur J Immunol. 2012 Mar;42(3):641–50.

20. Metenou S, Dembélé B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI, et al. Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population. J Immunol Baltim Md 1950. 2009 Jul 15;183(2):916–24.

21. Lyke KE, Dabo A, Arama C, Daou M, Diarra I, Wang A, et al. Reduced T regulatory cell response during acute Plasmodium falciparum infection in Malian children co-infected with Schistosoma haematobium. PloS One. 2012;7(2):e31647.

22. Dolo H, Coulibaly YI, Dembele B, Konate S, Coulibaly SY, Doumbia SS, et al. Filariasis attenuates anemia and proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults. PLoS Negl Trop Dis. 2012;6(11):e1890.

23. Hartgers FC, Obeng BB, Boakye D, Yazdanbakhsh M. Immune responses during helminth-malaria co-infection: a pilot study in Ghanaian school children. Parasitology. 2008 Jun;135(7):855–60.

24. Boef AGC, May L, van Bodegom D, van Lieshout L, Verweij JJ, Maier AB, et al. Parasitic infections and immune function: effect of helminth infections in a malaria endemic area. Immunobiology. 2013 May;218(5):706–11.

25. Wilson S, Jones FM, Mwatha JK, Kimani G, Booth M, Kariuki HC, et al. Hepatosplenomegaly associated with chronic malaria exposure: evidence for a pro-inflammatory mechanism exacerbated by schistosomiasis. Parasite Immunol. 2009 Feb;31(2):64–71.

26. Wilson S, Jones FM, Mwatha JK, Kimani G, Booth M, Kariuki HC, et al. Hepatosplenomegaly is associated with low regulatory and Th2 responses to schistosome antigens in childhood schistosomiasis and malaria coinfection. Infect Immun. 2008 May;76(5):2212–8.

27. Panda M, Sahoo PK, Mohapatra AD, Dutta S kanti, Thatoi PK, Tripathy R, et al. Decreased prevalence of sepsis but not mild or severe P. falciparum malaria is associated with pre-existing filarial infection. Parasit Vectors. 2013;6:203.

28. Wammes LJ, Hamid F, Wiria AE, de Gier B, Sartono E, Maizels RM, et al. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. Eur J Immunol. 2010 Feb;40(2):437–42.

29. Favre N, Ryffel B, Bordmann G, Rudin W. The course of Plasmodium chabaudi chabaudi infections in interferon-gamma receptor deficient mice. Parasite Immunol. 1997 Aug;19(8):375–83.

30. D'Ombrain MC, Robinson LJ, Stanisic DI, Taraika J, Bernard N, Michon P, et al. Association of early interferon-gamma production with

immunity to clinical malaria: a longitudinal study among Papua New Guinean children. Clin Infect Dis Off Publ Infect Dis Soc Am. 2008 Dec 1;47(11):1380–7.

31. Winkler S, Willheim M, Baier K, Schmid D, Aichelburg A, Graninger W, et al. Reciprocal regulation of Th1- and Th2-cytokine-producing T cells during clearance of parasitemia in Plasmodium falciparum malaria. Infect Immun. 1998 Dec;66(12):6040–4.

32. Luty AJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, et al. Interferon-gamma responses are associated with resistance to reinfection with Plasmodium falciparum in young African children. J Infect Dis. 1999 Apr;179(4):980–8.

33. Torre D, Speranza F, Giola M, Matteelli A, Tambini R, Biondi G. Role of Th1 and Th2 cytokines in immune response to uncomplicated Plasmodium falciparum malaria. Clin Diagn Lab Immunol. 2002 Mar;9(2):348–51.

34. Anosike JC, Onwuliri CO, Abanobi OC. A case of probable transplacental transmission of Wuchereria bancrofti microfilariae. Appl Parasitol. 1994 Nov;35(4):294–6.

35. Malhotra I, Ouma JH, Wamachi A, Kioko J, Mungai P, Njzovu M, et al. Influence of maternal filariasis on childhood infection and immunity to Wuchereria bancrofti in Kenya. Infect Immun. 2003 Sep;71(9):5231–7.

36. Soares Magalhães RJ, Biritwum N-K, Gyapong JO, Brooker S, Zhang Y, Blair L, et al. Mapping Helminth Co-Infection and Co-Intensity: Geostatistical Prediction in Ghana. PLoS Negl Trop Dis. 2011 Jun 7;5(6):e1200.

37. Malhotra I, Ouma JH, Wamachi A, Kioko J, Mungai P, Njzovu M, et al. Influence of maternal filariasis on childhood infection and immunity to Wuchereria bancrofti in Kenya. Infect Immun. 2003 Sep;71(9):5231–7.

38. van Riet E, Hartgers FC, Yazdanbakhsh M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. Immunobiology. 2007;212(6):475–90.

39. Hozo SP, Djulbegovic B, Hozo I. Estimating the mean and variance from the median, range, and the size of a sample. BMC Med Res Methodol. 2005;5:13.

40. Egger M, Schneider M, Davey Smith G. Spurious precision? Meta-analysis of observational studies. BMJ. 1998 Jan 10;316(7125):140–4.

41. Black N. Why we need observational studies to evaluate the effectiveness of health care. BMJ. 1996 May 11;312(7040):1215–8.

Appendix

Appendix 1: List and links of the 12 consulted online databases

Database	*URL*
PubMed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?otool=leiden
MEDLINE (OVID-version)	http://gateway.ovid.com/ovidweb.cgi?T=JS&MODE=ovid&NEWS=n&PAGE=main&D=prmz
Embase (OVID-version)	http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=main&MODE=ovid&D=oemezd
Web of Science	http://isiknowledge.com/wos
ScienceDirect	http://www.sciencedirect.com/
AIM (African Index Medicus)	http://indexmedicus.afro.who.int/
IMEMR (Index Medicus for the Eastern Mediterranean Region)	http://applications.emro.who.int/library/Databases/wxis.exe/Library/Databases/iah/?IsisScript=iah/iah.xic&base=imemr&lang=i

IMSEAR (Index Medicus for South-East Asia Region)	http://imsear.hellis.org/
IndMED	http://indmed.nic.in/
KoreaMED	http://koreamed.org/SearchBasic.php
LILACS (Latin America and the Caribbean)	http://lilacs.bvsalud.org/en/
WPRIM (Western Pacific Region Index Medicus)	http://www.wprim.org/

Appendix 2: keywords used for the search strategy

Database	*Query*
PubMed	("Helminthiasis"[Mesh] OR "helminthiasis"[all fields] OR "helminthiases"[all fields] OR "Nematomorpha Infection"[all fields] OR "Nematomorpha Infections"[all fields] OR "Cestode Infections"[all fields] OR "Diphyllobothriasis"[all fields] OR "Echinococcosis"[all fields] OR "Hymenolepiasis"[all fields] OR "Monieziasis"[all fields] OR "Taeniasis"[all fields] OR "Dictyocaulus Infections"[all fields] OR "Dirofilariasis"[all fields] OR "Fascioloidiasis"[all fields] OR "Monieziasis"[all fields] OR "Setariasis"[all fields] OR "Equine Strongyle Infections"[all fields] OR "Toxocariasis"[all fields] OR "Nematode Infections"[all fields] OR "Adenophorea Infections"[all fields] OR "Larva Migrans"[all fields] OR "Secernentea Infections"[all fields] OR "Trematode Infections"[all fields] OR "Clonorchiasis"[all fields] OR "Dicrocoeliasis"[all fields] OR "Echinostomiasis"[all fields] OR "Fascioliasis"[all fields] OR "Fascioloidiasis"[all fields] OR "Opisthorchiasis"[all fields] OR "Paragonimiasis"[all fields] OR "Schistosomiasis"[all fields] OR "Cestode Infection"[all fields] OR "Dictyocaulus Infection"[all fields] OR "Equine Strongyle

	Infection"[all fields] OR "Nematode Infection"[all fields] OR "Adenophorea Infection"[all fields] OR "Secernentea Infection"[all fields] OR "Trematode Infection"[all fields] OR "Sparganosis"[all fields] OR "Cysticercosis"[all fields] OR "Neurocysticercosis"[all fields] OR "Enoplida Infections"[all fields] OR "Enoplida Infection"[all fields] OR "Trichinellosis"[all fields] OR "Trichuriasis"[all fields] OR "Ascaridida Infections"[all fields] OR "Ascaridida Infection"[all fields] OR "Anisakiasis"[all fields] OR "Ascariasis"[all fields] OR "Ascaridiasis"[all fields] OR "Toxascariasis"[all fields] OR "Toxocariasis"[all fields] OR "Oxyurida Infections"[all fields] OR "Oxyurida Infection"[all fields] OR "Oxyuriasis"[all fields] OR "Enterobiasis"[all fields] OR "Rhabditida Infections"[all fields] OR "Rhabditida Infection"[all fields] OR "Strongyloidiasis"[all fields] OR "Spirurida Infections"[all fields] OR "Spirurida Infection"[all fields] OR "Dracunculiasis"[all fields] OR "Filariasis"[all fields] OR "Acanthocheilonemiasis"[all fields] OR "Dipetalonema Infections"[all fields] OR "Dipetalonema Infection"[all fields] OR "Dirofilariasis"[all fields] OR "Filarial Elephantiasis"[all fields] OR "Loiasis"[all fields] OR "Mansonelliasis"[all fields] OR "Onchocerciasis"[all fields] OR "Setariasis"[all fields] OR "Gnathostomiasis"[all fields] OR "Strongylida Infections"[all fields] OR "Hookworm Infections"[all fields] OR "Strongylida Infection"[all fields] OR "Hookworm Infection"[all fields] OR "Ancylostomiasis"[all fields] OR "Necatoriasis"[all fields] OR "Oesophagostomiasis"[all fields] OR "Strongyle Infections"[all fields] OR "Strongyle Infection"[all fields] OR "Trichostrongyloidiasis"[all fields] OR "Dictyocaulus Infections"[all fields] OR "Dictyocaulus Infection"[all fields] OR "Haemonchiasis"[all fields] OR "Ostertagiasis"[all fields] OR "Trichostrongylosis"[all fields] OR "Neuroschistosomiasis"[all fields] OR "Helminths"[Mesh] OR "Helminths"[all fields] OR "Helminth"[all fields] OR "Parasitic Worms"[all fields] OR "Parasitic Worm"[all fields] OR "Aschelminthes"[all fields] OR "Aschelminthe"[all fields] OR "Gordius"[all fields] OR "Nematomorpha"[all fields] OR "Nematomorphas"[all fields] OR "Acanthocephala"[all fields] OR "Moniliformis"[all fields] OR "Nematoda"[all fields] OR "Adenophorea"[all fields] OR "Secernentea"[all fields] OR "Platyhelminths"[all fields] OR "Cestoda"[all fields] OR "Trematoda"[all fields] OR "Turbellaria"[all fields] OR "Rotifera"[all fields] OR "Enoplida"[all fields] OR "Dioctophymatoidea"[all fields] OR "Mermithoidea"[all fields] OR "Trichuroidea"[all fields] OR "Capillaria"[all fields] OR "Trichinella"[all fields] OR "Trichinella spiralis"[all fields] OR "Trichuris"[all fields] OR "Ascaridida"[all fields] OR "Ascaridia"[all fields] OR "Ascaridoidea"[all fields] OR "Anisakis"[all fields] OR "Ascaris"[all fields] OR "Ascaris

	lumbricoides"[all fields] OR "Ascaris suum"[all fields] OR "Toxascaris"[all fields] OR "Toxocara"[all fields] OR "Toxocara canis"[all fields] OR "Oxyurida"[all fields] OR "Oxyuroidea"[all fields] OR "Enterobius"[all fields] OR "Rhabditida"[all fields] OR "Rhabdiasoidea"[all fields] OR "Strongyloides"[all fields] OR "Rhabditoidea"[all fields] OR "Caenorhabditis"[all fields] OR "Spirurida"[all fields] OR "Camallanina"[all fields] OR "Dracunculoidea"[all fields] OR "Dracunculus Nematode"[all fields] OR "Spirurina"[all fields] OR "Filarioidea"[all fields] OR "Acanthocheilonema"[all fields] OR "Brugia "[all fields] OR "Dipetalonema"[all fields] OR "Dirofilaria "[all fields] OR "Loa"[all fields] OR "Mansonella"[all fields] OR "Microfilaria"[all fields] OR "Onchocerca "[all fields] OR "Setaria Nematode"[all fields] OR "Wuchereria"[all fields] OR "Spiruroidea"[all fields] OR "Thelazioidea"[all fields] OR "Gnathostoma"[all fields] OR "Strongylida"[all fields] OR "Ancylostomatoidea"[all fields] OR "Ancylostoma"[all fields] OR "Necator"[all fields] OR "Necator americanus"[all fields] OR "Heligmosomatoidea"[all fields] OR "Nematospiroides"[all fields] OR "Nematospiroides dubius"[all fields] OR "Nippostrongylus"[all fields] OR "Metastrongyloidea"[all fields] OR "Angiostrongylus"[all fields] OR "Molineoidae"[all fields] OR "Nematodirus"[all fields] OR "Strongyloidea"[all fields] OR "Oesophagostomum"[all fields] OR "Strongylus"[all fields] OR "Trichostrongyloidea"[all fields] OR "Dictyocaulus"[all fields] OR "Haemonchus"[all fields] OR "Ostertagia"[all fields] OR "Trichostrongylus"[all fields] OR "Tylenchida"[all fields] OR "Tylenchoidea"[all fields]) AND ("Malaria"[Mesh] OR malaria* OR "malaria"[all fields] OR "Paludism"[all fields] OR "Plasmodium Infections"[all fields] OR "Plasmodium Infection"[all fields]) AND ("Immunity, Cellular"[Mesh] OR "cellular immune response"[all fields] OR "cellular immune responses"[all fields] OR "Cellular Immunities"[all fields] OR "Cellular Immunity"[all fields] OR "Cell-Mediated Immunity"[all fields] OR "Cell Mediated Immunity"[all fields] OR "Cell-Mediated Immunities"[all fields] OR "Antigen Presentation"[all fields] OR "Immunologic Surveillance"[all fields] OR "Lymphocyte Activation"[all fields] OR "Cross-Priming"[all fields] OR "Cytokines"[mesh] OR "cytokines"[all fields] OR "cytokine"[all fields] OR "CD4-Positive T-Lymphocytes"[Mesh] OR "Antigens, CD4"[Mesh] OR "cd4"[all fields] OR "cd-4"[all fields] OR "t cell"[all fields] OR "t cells"[all fields] OR "T-Lymphocytes"[Mesh] OR "T-Lymphocyte"[all fields] OR "T-Lymphocytes"[all fields] OR "b cell" OR "b cells" OR "B-Lymphocytes"[Mesh] OR "B-Lymphocyte"[all fields] OR "B-Lymphocytes"[all fields] OR "Dendritic Cells"[Mesh] OR "Dendritic Cells"[all fields] OR "Dendritic Cell"[all fields] OR "Chemokines"[all fields] OR "Chemokine"[all fields] OR "beta-

	Thromboglobulin"[all fields] OR "Macrophage Inflammatory Proteins"[all fields] OR "Growth Differentiation Factor"[all fields] OR "Growth Differentiation Factors"[all fields] OR "Hematopoietic Cell Growth Factors"[all fields] OR "Hematopoietic Cell Growth Factor"[all fields] OR "Haematopoietic Cell Growth Factors"[all fields] OR "Haematopoietic Cell Growth Factor"[all fields] OR "Colony-Stimulating Factors"[all fields] OR "Colony-Stimulating Factor"[all fields] OR "Stem Cell Factor"[all fields] OR "Stem Cell Factors"[all fields] OR "Hepatocyte Growth Factor"[all fields] OR "Hepatocyte Growth Factors"[all fields] OR "Interferons"[all fields] OR "Interferon"[all fields] OR "Interleukin"[all fields] OR "Interleukins"[all fields] OR Interleukin*[all fields] OR "Leukemia Inhibitory Factor"[all fields] OR "Leukemia Inhibitory Factors"[all fields] OR "Lymphokines"[all fields] OR "Lymphokine"[all fields] OR "Leukocyte Migration-Inhibitory Factors"[all fields] OR "Leukocyte Migration-Inhibitory Factor"[all fields] OR "Lymphotoxin-alpha"[all fields] OR "Macrophage-Activating Factors"[all fields] OR "Macrophage Migration-Inhibitory Factors"[all fields] OR "Macrophage-Activating Factor"[all fields] OR "Macrophage Migration-Inhibitory Factor"[all fields] OR "Transfer Factor"[all fields] OR "Transfer Factors"[all fields] OR "Monokines"[all fields] OR "Monokine"[all fields] OR "Tumor Necrosis Factor"[all fields] OR "Tumor Necrosis Factors"[all fields] OR "Tumour Necrosis Factor"[all fields] OR "Tumour Necrosis Factors"[all fields] OR "Oncostatin M"[all fields] OR "Osteopontin"[all fields] OR "Transforming Growth Factor"[all fields] OR "Transforming Growth Factors"[all fields] OR "4-1BB Ligand"[all fields] OR "CD70"[all fields] OR "B-Cell Activating Factor"[all fields] OR "B-Cell Activating Factors"[all fields] OR "CD30"[all fields] OR "CD40"[all fields] OR "Ectodysplasins"[all fields] OR "Fas Ligand Protein"[all fields] OR "Fas Ligand Proteins"[all fields] OR "Lymphotoxin-alpha"[all fields] OR "Lymphotoxin-beta"[all fields] OR "OX40 Ligand"[all fields] OR "RANK Ligand"[all fields] OR "TNF"[all fields] OR "dendritic"[all fields]) NOT ("Animals"[mesh] NOT "Humans"[mesh])
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Chapter 8

General discussion

What we already knew

Helminth-induced modulation of *Plasmodium sp*. immune responses: What have we learnt from population based studies?

Studies using animal models have been pivotal to understand the effect of helminths on their host immune responses. In general they indicated that chronic helminth infections had the potential to skew the immune response toward a Th2 profile and to trigger the stimulation of a powerful regulatory network (1). Interestingly these immune modifications have been shown to lead to alterations of the host immune response to helminths in the first place, but also to others pathogens such as *Plasmodium sp.* (2). The implication of such findings is enormous given the geographical distribution of malaria worldwide. During the past years efforts have been put into epidemiological studies to verify these findings in affected populations. With regard to the humoral response, the main hypothesis was that in the context of coinfection the antibodies against *P. falciparum* which is dominated by cytophilic antibodies (IgG1 and IgG3) would be altered by the Th2 skewing of immune response observed in those chronically infected with helminths characterized by the predominance of IgG4 and IgE. To date only few studies have addressed this. Two of these studies were conducted in Senegal. The first one by Diallo *et al.* showed that IgG1 and IgG3 antibodies specific to PfMSP1-$_{19}$ and to *P. falciparum* schizont extract were significantly increased in children co-infected with *S. haematobium* and *P. falciparum* by comparison to those with *P. falciparum* mono infection only (3). Interestingly neither IgG2, IgG4 nor IgE levels specific to the same antigens were different between the two groups compared. These observations suggest that *S. haematobium* favours an efficient anti-malaria protective antibody response despite its Th2 skewing. In the second study the opposite observation was made by Roussilhon and co-workers (4). They showed that worm carriage was significantly correlated, on the one hand, with a decrease in cytophilic IgG1 and IgG3 antimalarial antibodies and, on the other, with an increase of non cytophilic IgG4 antibodies. Finally in a third study conducted in Mali no difference was observed between co-infected and mono-infected subjects in terms of their humoral response (5). These discrepancies in results obtained might indicate differences

in the study methodology or numerous environmental factors but most importantly indicates the need for further studies.

Antibody response although important is not enough to clear malaria infection on its own. Studies have shown that in contrast to normal mice, mice that are deficient in T cells cannot mount a sufficient immune response to *P. yoeli* infection after passive transfer of *Plasmodium* specific antibodies (6). These mice have a delayed time to appearance of microscopic parasitemia but cannot completely clear the parasite. This indicates that the humoral response might be important at the onset of the infection to limit the progression of the disease but that a complete clearance of the parasite is cell mediated. Cell mediated immunity specific to *Plasmodium spp.* has been shown to be complex and to involve a wide range of interacting innate and adaptive responses. Controlled human infection of malaria naive volunteers with *P. falciparum* has shown that both innate and adaptive immune cells contribute to the production of IFNγ a major pro-inflammatory cytokine (7,8) marking the so called Th1 response important for parasite clearance (9). Although in these studies no data were provided on the Th2 and regulatory responses the importance of the Th2 response in anti-malarial immunity as well as the need for an efficient regulatory response to avoid tissue damage has been suggested (reviewed in 10). There is evidence that some of the innate and adaptive effector mechanisms involved in immunity to malaria are modulated by helminths. For example in a study conducted in Sudanese children, it was observed that *S. haematobium* and *S. mansoni* were able to significantly impair the function of NK cells, a major source of IFNγ (11). In addition subjects chronically infected with helminths have been shown to have a lower frequency of dendritic cells (DC) which, also have an impaired function as indicated by their reduced capacity to respond to TLR ligands and to activate T cells (12,13). With regard to adaptive immune responses, it is now known that chronic helminth infections are linked to T cell hyporesponsivness mediated either through intrinsic mechanisms (14) or via their ability to induce regulatory cells that inhibit T cell responses (15–17). All these mechanisms are in the first place for helminths to evade the immune system, but as a off target effect may interfere with anti-malarial immunity.

In the context of coinfection it has been suggested that the anti-inflammatory milieu induced by chronic helminth infections may dampen both the protective and immunopathological immune responses to *Plasmodium spp*.. As a consequence subjects living in endemic areas would be more likely to acquire new malaria infection but will develop fewer symptoms or have less severe disease. Researchers have tried to test this hypothesis in naturally exposed individuals who live in areas endemic for both parasites. From a clinical point of view, some studies reported that helminths could exert either a protective (18–20) or an aggravating effect (21,22) on malaria outcomes while others indicated no association (23). Finally whether the cell mediated immune response specific to *Plasmodium spp.* was affected by a concurrent helminth infection has also been assessed during the past few years. The studies looked at the cytokine profile of co-infected subjects and compared it to *Plasmodium spp.* mono-infected ones. They showed an increase (24,25), a decrease (26,27) or even an absence (28) of effect of chronic helminth infections on the levels of Th1, Th2 or regulatory cytokines in coinfected subjects. Several reasons were suggested to explain the observed differences such as methodology, study design, helminths species, environmental factors (29) and again, more data are needed to draw a definitive conclusion.

Key points regarding what we already knew:

Helminths and malaria overlap in their geographical distribution.

Chronic helminth infections induce a Th2 skewed immune response and a potent immune regulatory network in chronically infected subjects.

Helminths induced immune modulation can interfere with *Plasmodium spp.* specific immune response in mouse models.

Epidemiological studies show variable effect of helminths on malaria outcome and immune response to malaria parasites.

What this thesis adds to our knowledge

Chronic *S. haematobium* infection leads to an alteration of B cells function and changes in the distribution of B cell subsets

The role of B cells in immunity against pathogens is being actively examined and is currently shown to expand beyond the classical antibody production. B cells are at the intersection between innate and adaptive immune response. They can produce cytokines upon stimulation through pathogens recognition receptor (PRR) and B cell receptors (BCR) and activated B cells can act as antigen presenting cells (APC) and are able to deliver co-stimulatory signals leading to the differentiation of Th2 effector cells. Moreover in recent studies they have been recognized for their immune regulatory properties (reviewed in (30)). The multitude of B cell functions are mediated through a variety of subsets with distinct phenotypic and functional characteristics, however, data are still scarce on the extent to which their distribution and function are altered in subjects bearing chronic infection. This research question was addressed in a field study described in chapter 3. B cell subsets and B cell responses to BCR and Toll-like receptor 9 stimulation was assessed in infected school aged children and in their uninfected counterparts at baseline and 6 months after the removal of the trematodes by Praziquantel. We observed that while the percentage of total B cells was similar between infected and uninfected individuals the distribution of some B cell subsets was significantly altered by active *S. haematobium* infection. This alteration was characterised by a trend toward a decrease of the naive B cell population in infected individuals that was significantly increased upon praziquantel treatment. On the other hand, we noted an increased percentage of activated and atypical B cells in the schistosoma infected group along with an increase of double negative and switched memory B cells subsets. These findings should be interpreted to be in line with data suggesting that atypical and double negative memory B cells are increased in chronic infection or disease and are functionally impaired (31–33) . Recently, the function of atypical MBC was assessed in relation to classical MBC in Malian adults with lifelong exposure to malaria and Malian children developing clinical malaria (33). It was observed that in malaria exposed and infected individuals atypical MBC differentiated from classical MBC and these cells were

characterized by the expression of an array of inhibitory receptors and by an impairment of BCR signalling. These changes were associated with an impaired B cell proliferation, cytokine production and antibody secretion upon stimulation. In our study we noted some functional changes as well in that the total B cell population of *S. haematobium*-infected children had a lower expression of Ki-67, a marker of cell proliferation, and produced less TNF, somehow resembling what might be considered as an exhausted profile. Taken together these findings might indicate that as expected infection with *S. haematobium* leads to B cells activation and immunoglobulin class switching of B cells. However in chronically infected individuals the repeated stimulation of B cells results in a state of B cells exhaustion or hyporesponsiveness that is partially restored upon specific anti-schistosoma treatment. This phenomenon might help the parasite to prolong it survival within the definitive host and explain why acquisition of protective humoral immunity takes time to develop in individuals living in endemic areas. It can also contribute towards limiting tissue damage in the host. However it is still unclear whether and how this down regulation of the B cell response due to *S. haematobium* infection can also affect the host response to other pathogens.

Individuals chronically infected with helminths have an impaired antibody response to *P. falciparum* sexual stage antigens

Sexual stage of *Plasmodium spp.* plays a central role in malaria transmission. Epidemiological studies have indicated that helminth co-infected individuals are more likely to carry *P. falciparum* gametocyte (34). Unfortunately not much is known about the mechanisms underlying such association. Gametocytogenesis can be influenced by various stress-inducing factors including the *Plasmodium* specific immune responses (reviewed in (35)). On the other hand helminths are well known for their ability to modulate the immune system of their host and question might arise as to whether in coinfected individuals this helminth induced immune modulation might interfere with anti-gametocyte immunity. This question was addressed for the first time in a cross-sectional study described in chapter 4. Our principal objective was to determine the influence of *S. haematobium* and filaria co-infection with malaria parasite on the antibody responses to *P. falciparum* sexual stage antigens. The study was conducted in an area

endemic for both helminths and for malaria as described in chapter 2. Total IgG to sexual stage antigens Pfs230 and Pfs48/45 was measured and their levels compared between individuals coinfected with helminths and *P. falciparum* or with only with *P. falciparum.* It was observed that IgG to Pfs48/45 sexual stage antigen but not Pfs230 was significantly lower in co-infected individuals even after adjustment for confounding factors such as age, haemoglobin level or the density of *P. falciparum* asexual stage parasite. This is the first time that helminth infection is reported to influence an immunological response to a *P. falciparum* sexual stage antigen. Additional studies will be needed to test this finding in different geographical areas. If confirmed, this could have implications for malaria transmission. Indeed anti Pfs48/45 IgG is known to exert transmission blocking activity by inhibiting the fertilisation of *P. falciparum* gametes in the mosquito gut (36). Hence a lower antibody response to this antigen due to helminths might result in an increased multiplication of *P. falciparum* in the mosquito gut and an increase of its transmission to humans. This hypothesis will need to be tested in future studies.

From the two helminth species considered in this study only *S. haematobium* but not filarial parasites was associated with a significant decrease in antibodies to *P. falciparum* sexual stage antigens. Although this has to be confirmed in further studies, we hypothesize that while *L. loa*, *M. perstans* and *S. haematobium* are all located in the blood vasculature of their host, their effect on the immune response or on host metabolism might be more pronounced for *S. haematobium*. For example, anaemia was more frequent in subjects infected with *S. haematobium*. The selective effect of *S. haematobium* infection on Pfs48/45 and not Pfs230 is difficult to explain. It is possible that the modulatory effect exerted by helminths is not sufficient to impair the humoral response to Pfs230 antigens. Data from field studies (35,36) have shown Pfs230 to be more immunogenic than Pfs48/45, however, another explanation would be that our study was underpowered to detect such an effect.

Despite the fact that *S. haematobium* infection was associated with an impaired response to Pfs48/45 antigens, we did not observe a decrease in gametocyte carriage in subjects infected with schistosoma and/or filarial parasites. This suggests that Pfs48/45 plays a limited role in the

development of gametocytes within the human host. This is supported by a study where sera from individuals naturally exposed to *P. falciparum* were used to assess the capacity of sexual stage antigens to induce complement-mediated lysis of *P. falciparum* gametes (37). In the study, antibodies to Pfs230 but not Pfs48/45 were positively associated with complement-mediated lysis of *P. falciparum* gametes *in vitro*. Interestingly they observed that Pfs230-induced gamete lysis was more pronounced in the presence of Pfs48/45.

Our study presented some limitations that need to be addressed. For example, a longitudinal study design would have been more appropriate, it will take into account the effect of seasonality on gametocytogenesis (38,39) and will allow the dynamic study of the association between helminths, *P. falciparum* sexual stage carriage and the immune response. Furthermore gametocyte carriage was determined by microscopy which has proven to be less sensitive than PCR to detect low parasite load (38). Finally functional assays such as Standard Membrane Feeding assays (SMFA) are instrumental to assess whether the decrease of sexual stage antibodies translate into an increased transmission of *P. falciparum* to the mosquito and should be included in future studies. Despite these limitations our study brings novel information on the complex interaction between helminths and malaria and generates a hypothesis that needs to be further tested.

Antibody responses to *P. falciparum* asexual stage antigens are not influenced by either *S. haematobium* or filarial infections

To date only few studies have assessed whether chronic helminth infections could influence carriage and antibody responses to asexual stages of *P. falciparum*. So far such studies have generated contradictory outcomes. This indicates the need for additional data and gives justification for the study described in chapter 4. This study was conducted in an area where malaria and helminths are highly endemic despite regular mass drug administration. It aimed at assessing the relationship between helminth infection, carriage of *P. falciparum* trophozoites and antibody responses to asexual stage antigens. Our first observation was that the asymptomatic carriage of *P. falciparum* was quite high (42% by microscopy and 75% by PCR) and was accompanied by a similarly high prevalence of *S. haematobium* (75%)

in the area. Moreover individuals infected with *S. haematobium* had a higher carriage of *P. falciparum* asexual forms as determined by microscopy. Interestingly this association disappeared when *P. falciparum* carriage was assessed by PCR. These findings suggest that schistosoma infected and uninfected individuals are equally susceptible to *P. falciparum* infection however higher parasite replication seems to be favoured in the presence of *S. haematobium*. Similar increase of microscopic carriage of asexual form of *P. falciparum* in helminth infected individuals have been reported elsewhere (40,41). However there are also studies where a negative association was reported (42,43). The difference in the studies might be due to confounding factors such as the characteristic of the study area, differences in study methodology, malaria seasonality, the type of helminths species assessed as well as the intensity and chronicity of helminth infections.

Despite the higher density of *P. falciparum* detected by microscopy in *S. haematobium* coinfected subjects we failed to link this to an impairment of the IgG response to asexual stage antigens (MSP1, AMA1 and GLURP). When considering published data, we noted that only one study have so far reported an increase in antibodies specific to asexual stage antigens in *S. haematobium* coinfected individuals (12). In this study, subjects free of schistosome infection who served as the comparator group to those with both schistosomes and malaria were selected from an area not endemic for helminths. This might have represented a bias since confounding factors such as differences in the living environment were not controlled for. Other population studies in which helminth infected and uninfected subjects were recruited in the same area showed a decrease of the IgG response to *P. falciparum* in helminths coinfected individuals (44) or even an absence of effect of helminths (45).

These differences reflect the complex interaction that exists between helminths and malaria parasite. It could indicate that in individuals living in affected areas the modulation induced by helminths on malaria specific humoral response might be confounded by the degree of endemicity as well as by past helminth infections. It is also possible that the modulatory effect of helminths that favour *P. falciparum* replication is exerted through different mechanisms. For example through T cells hypo responsiveness or possibly through metabolic changes. Future studies would need to consider both the humoral and

cellular immune response of the study participants. It would also be important to determine whether helminths are capable of favouring the switch from asymptomatic to symptomatic *P. falciparum* infection. This was not possible in this study considering its cross sectional design.

P. falciparum **infection imposes a distinct signature on the cytokine and chemokine profiles, which is not impaired by a concurrent infection with** ***S. haematobium***

The innate and adaptive cellular immune responses play an important role in anti-malarial immunity (46). They serve to control the infection but at the same time can also be detrimental to the host by causing tissue damage (47). Several studies have been conducted in individuals naturally exposed to *Plasmodium sp.* to characterize their cytokine response when they are infected. These studies mainly focus on few cytokines and in addition their data analysis does not take into consideration the level of correlation that might exist among the measured cytokines. This is rather an important aspect given that from a functional perspective cytokines can have a synergistic (48,49) or antagonist effect (50). In chapter 6 we set out to assess the effect of *P. falciparum* and *S. haematobium* infections on the cytokine response of infected individuals. We assessed both the innate and adaptive immune responses using in vitro assays where cytokines and chemokines were measured after PBMC or whole blood stimulation with either SEB (to assess the adaptive immune response) or various TLRs ligands (to assess the innate immune response). Moreover instead of looking at individual cytokine s, we opted for "Principal Component Analysis" (PCA), which allows a simultaneous assessment of multiple variables that are then summarized into new synthetic variables or components containing most of the information in the dataset. An additional advantage of this PCA is that the synthetic components also take into consideration the magnitude of the correlation between the different cytokines/chemokines. What we observed when we compared the different principal components among the study groups is that; *P. falciparum* infection was associated with an increase of the general immune responsiveness of the study subjects. This increase was seen both for the innate and the adaptive immune system and was not impaired by a concurrent *S. haematobium* infection despite its ability to

decrease the pro-inflammatory immune response as observed in *S. haematobium* singly infected subjects. From an immunological point of view our finding might indicate that although schistosomes have the potential to modulate the immune response, the observed immune modulation is not sufficient to dampen the cell mediated immune response triggered by the presence of *P. falciparum* in the blood stream. It is possible that malaria parasites, seen as an acute infection by the host immune system, overcome the threshold set by the regulatory network induced by helminths. Another explanation for lack of an effect of helminth infections on cytokine response to malaria infection could be the epidemiological feature of the study area as well as on the characteristic of the study population. It is possible that the subjects that appear free of a particular helminth infection have been infected in the past or carry other helminths species capable of shaping the host immune system. Our study participants were recruited in an area where the prevalence of helminths has been shown to be consistently high (51) and although we did not assess whether they had past helminths infection, it is very likely that they were infected in the past. In this regard in chapter 5 we observed that regulatory T cells of new-borns from filaria infected mothers correlated negatively with the level of pro-inflammatory Th1 cells suggesting that regulatory T cells acquired functional capacity already before birth probably as a consequence of *in utero* exposure to helminths. If the in utero effect of helminth infections would be long-lived, these data would be in line with the notion that early life exposure to helminths might make it difficult to see any clear effect of current helminth infection on various immune responses.

Although poly-helminth infections are the norm in tropical and sub tropical areas (52,53), epidemiological studies where the effect of helminths on anti-malaria immunity was assessed have mainly focused on one or maximum two helminth species. This could be responsible for the disparities in outcomes. The influence of poly helminth infection as well as past helminth infections on the outcomes of the responses studied was further assessed in the meta-analysis describe in chapter 7. This meta-analysis indicates that there are currently 19 eligible studies conducted in endemic areas to assess the influence of helminths on immune responses of *P. falciparum* infected subjects. The results seem highly conflicting. However when these studies are pooled together within a meta-analysis, the emerging trend was that the cell

mediated immune response to *P. falciparum* was not affected by helminths in coinfected individuals. Interestingly when we stratified our assessment using a moderator analysis we noted that studies where stringent criteria were used, to control for the confounding effect of past exposure to helminth antigens, showed that in those who have no current or past exposure to helminths, there is significantly higher pro-inflammatory cytokines in malaria infected subjects (54,55). Additionally this moderator analysis indicated that poly-helminth infections with schistosomes and intestinal helminths resulted in a significant increase of regulatory cytokines (56). These findings support the results described in chapter 6 and further indicate that interaction between helminths and malaria is complex. It is indeed possible that the helminth induced modulation of their host immune system has the potential to interfere with their cell mediated immune response to *P. falciparum*. However in areas where the force of infection with helminths is high, this effect could be masked by past and/or current (poly) helminth infections.

The type of immunological assays used in epidemiological studies might influence outcomes

When we conducted the meta-analysis described in chapter 7 we noticed that our current knowledge of malaria and helminth co-infection was based on studies that used different immunological assays. For example some studies report on circulating plasma or serum cytokines (25,26,57,58) whereas others measure cytokines after stimulation of whole blood (27,59–62) or PBMC (63) either in supernatants or intra-cellularly. In addition the type of stimuli used when cells are stimulated can also be different between studies. These differences may contribute to the heterogeneity of the results observed in different studies. Indeed when performing a moderator analysis aiming to understand the variation in study outcomes we noted that the Th17 response was higher in helminth and malaria co-infected individuals when this cytokine was measured by flow cytometry after intracellular staining (27). Moreover studies where cells were stimulated with PHA (64) or *P. falciparum*-infected red blood cells (iRBC) (27,59,61), were more likely to report a higher Th2 or anti-inflammatory response in helminths and malaria co-infected individuals compared to those with malaria only. These observations underline the

importance of statistical method such as meta-analysis to account for the heterogeneity in study design when interpreting results from population based studies. Moreover they also indicate the need for standard operating procedures to harmonize the protocols used in field studies.

Key points on what this thesis adds to our knowledge

Regulatory T cells of a newborn from helminth-infected mother can suppress the development of effector T cells probably as a result of *in-utero* exposure to helminths.

Helminths can alter B cell profile and function but in particular lead to expansion of atypical and double negative B cells.

Helminths can influence antibody response to *P. falciparum* sexual stage antigens.

Individuals chronically infected with helminths do not have an impaired IgG response to *P. falciparum* asexual stage antigens.

P. falciparum leads to a general increase of the innate and adaptive immune response in infected individuals.

The innate and adaptive cytokine responses of individuals infected with *P. falciparum* is not altered by a concurrent *S. haematobium* infection.

The absence of an effect of helminths on the cellular immune response of individuals with malaria is confirmed by a meta-analysis of all the studies available to date.

Past infection with helminths or current poly-helminth infections can mask the effect of a helminth on *P. falciparum* immune response.

There is substantial degree of heterogeneity in studies that assess the association between helminths and malaria. Standardized and uniform protocols are needed to be able to answer whether helminth infections affect immune responses to *P. falciparum*.

Direction for future research

Helminths and malaria transmission

Indirect evidence suggests a role for helminths in sustaining malaria transmission. However to date very few studies have addressed this question. In this thesis we showed that *S. haematobium* was able to decrease antibody responses to *P. falciparum* sexual antigens. This might lead to an overall impairment of the transmission blocking immunity. Whether this finding could result in a significant increase of malaria transmission in a given area would need to be addressed in future studies. These studies might also need to use approaches such as Standard Membrane Feeding Assays (SMFA) to assess the transmission blocking activity of these antibodies or ecological studies to determine whether malaria incidence, or asymptomatic carriage of sexual and asexual stage *P. falciparum* correlate with the prevalence/burden of helminths in the population.

Helminths and malaria vaccine

Malaria vaccines bear a lot of promise for the eradication or at least the control of malaria in affected areas. To date more than 150 malaria vaccine candidate are or were undergoing clinical evaluation (65) and only one candidate vaccine, the RTS'S has reached a phase III where it was shown to confer modest protection against malaria (66). Data are still scarce as to whether helminths could impair or augment the response to these candidate vaccines in endemic populations. A study recently showed that the immunogenicity of the GMZ2-4 blood stage malaria vaccine candidate was significantly reduced in 1 to 5 years old children who were infected with *Trichuris trichiura* (67). Similar studies need to be repeated. If this effect is confirmed, understanding the mechanism of this interaction could be of importance in the design of malaria vaccine and immunization program. A particular attention should also be given to malaria transmission blocking vaccine candidates such as Pfs48/45 antigen given that the antibody response elicited has been shown to be impaired by helminths.

Beyond helminths: Rural VS urban differences

Rapid urbanization is taking place in Africa. In western countries an increase of urbanization index has been linked with a decrease of biodiversity and emergence of inflammatory diseases (68). Such an alteration of the biodiversity might be reflected in differences in immune responses of subjects from different areas. This was recently shown by Mbow *et al.* in a study where they compare the immune profile of subjects living in rural Africa to those of individuals from urban Africa and Europe (69). What they observed was a rural to urban gradient marked by a higher Th1 and Th2 and markedly a Th2/Th1 ratio in subjects from rural Africa by comparison to those from urban Africa and Europe. Similar rural and urban differences were also observed between individuals from urban Africa and Europe and when memory T cells subsets were assessed (69). In a different study Smolen *et al.* showed that innate cytokine response of 2 years old children measured after stimulation of whole blood cells with TLR ligands were similar between children from North America, South America and Europe despite obvious differences in their living environment (70). Yet children from South Africa presented a different profile. Difference in innate response between African and Europeans children was reported in a study by Labuda et al. (71). How geographical differences in immune response of rural and urban dwellers can affect response to malaria parasites or how this affects interaction between helminths and malaria parasites is not yet known. Filling this gap in our knowledge will be of particular importance in the future, since according to the United Nation nearly 50% of African population will be leaving in urban cities by 2035.

Beyond helminths: Microbiome and malaria

Additional effort should be invested in assessing the relationship between *P. falciparum* and the microbiome of their host, particularly the gut microbiota. A recent study indicated that the gut microbiome could protect their human host against *P. falciparum* through antibody responses against the Galα1-3Galβ1-4GlcNAc-R glycan (also known as alpha-gal) (72). This xeno-glycan has been shown to be expressed both by bacteria of the gut flora and by *Plasmodium sp.* parasite. In this study the protective effect of the microbiome was shown to be

mediated through the production of anti-alpha-gal antibodies capable of blocking *Plasmodium sp*. growth in vitro through complement activation and to interfere with the invasion of hepatocytes by the parasite (72). It is possible that additional xeno-glycans commonly expressed by gut bacteria and by *Plasmodium sp.* are able to generate such an anti-malarial immunity. Their identification and the characterization of the mechanism by which they are able to modulate anti-malaria immunity could open the door to a new era of glycan based anti-malaria vaccines. Whether the composition of the microbiome could influence the immunogenicity and efficacy of malaria vaccine candidates would also need to be addressed. This has been shown for influenza vaccine in mice (73). Moreover the interaction of helminths with malaria might need to take the microbome into account as it has been shown that helminths can influence the gut microbiome (74).

Age might matter

Studies that describe the epidemiology of helminths and malaria coinfection have mainly focused on school aged children and adults to some extent. There is currently an important gap in our knowledge on co-infection in pre-school age children. However there are data suggesting that this group is also affected. In a exploratory studies where the burden of helminths was assessed in 1 to 5 years old children in Gabon we found a prevalence of 29% of intestinal helminths (*Ascaris lumbricoides, Trichuris trichiura*, *Necator Americanus*) and 7% of *S. haematobium* (unpublished data*)*. It is likely that helminths have a more potent effect on anti-malaria immunity in this age group.

Standard operating procedure for immuno-epidemiological studies

In this thesis we were able to show that the differences in immunological assays set up to study the effect of helminths on immune responses to *Plasmodium spp.* infection influence the direction of the study outcome. Given the importance of immuno-epidemiological studies, there is a need to harmonize the protocols used or at least to define some strict guidelines.

References

1. Hoerauf A, Satoguina J, Saeftel M, Specht S. Immunomodulation by filarial nematodes. Parasite Immunol. 2005 Nov;27(10-11):417–29.

2. Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. Helminth parasites--masters of regulation. Immunol Rev. 2004 Oct;201:89–116.

3. Diallo TO, Remoue F, Gaayeb L, Schacht A-M, Charrier N, De Clerck D, et al. Schistosomiasis coinfection in children influences acquired immune response against Plasmodium falciparum malaria antigens. PloS One. 2010;5(9):e12764.

4. Roussilhon C, Brasseur P, Agnamey P, Pérignon J-L, Druilhe P. Understanding human-Plasmodium falciparum immune interactions uncovers the immunological role of worms. PloS One. 2010;5(2):e9309.

5. Lyke KE, Wang A, Dabo A, Arama C, Daou M, Diarra I, et al. Antigen-specific B memory cell responses to Plasmodium falciparum malaria antigens and Schistosoma haematobium antigens in co-infected Malian children. PloS One. 2012;7(6):e37868.

6. Roberts DW, Rank RG, Weidanz WP, Finerty JF. Prevention of recrudescent malaria in nude mice by thymic grafting or by treatment with hyperimmune serum. Infect Immun. 1977 Jun;16(3):821–6.

7. Roestenberg M, Teirlinck AC, McCall MBB, Teelen K, Makamdop KN, Wiersma J, et al. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. Lancet. 2011 May 21;377(9779):1770–6.

8. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJF, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. N Engl J Med. 2009 Jul 30;361(5):468–77.

9. Inoue S-I, Niikura M, Mineo S, Kobayashi F. Roles of IFN-γ and γδ T Cells in Protective Immunity Against Blood-Stage Malaria. Front Immunol. 2013;4:258.

10. Perez-Mazliah D, Langhorne J. CD4 T-cell subsets in malaria: TH1/TH2 revisited. Front Immunol. 2014;5:671.

11. Feldmeier H, Gastl GA, Poggensee U, Kortmann C, Daffalla AA, Peter HH. Relationship between intensity of infection and immunomodulation in human schistosomiasis. II. NK cell activity and in vitro lymphocyte proliferation. Clin Exp Immunol. 1985 May;60(2):234–40.

12. Everts B, Adegnika AA, Kruize YCM, Smits HH, Kremsner PG, Yazdanbakhsh M. Functional impairment of human myeloid dendritic cells during Schistosoma haematobium infection. PLoS Negl Trop Dis. 2010;4(4):e667.

13. Semnani RT, Liu AY, Sabzevari H, Kubofcik J, Zhou J, Gilden JK, et al. Brugia malayi microfilariae induce cell death in human dendritic cells, inhibit their ability to make IL-12 and IL-10, and reduce their capacity to activate CD4+ T cells. J Immunol Baltim Md 1950. 2003 Aug 15;171(4):1950–60.

14. Taylor JJ, Krawczyk CM, Mohrs M, Pearce EJ. Th2 cell hyporesponsiveness during chronic murine schistosomiasis is cell intrinsic and linked to GRAIL expression. J Clin Invest. 2009 Apr;119(4):1019–28.

15. Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, et al. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis. 2012;6(5):e1655.

16. García-Hernández MH, Alvarado-Sánchez B, Calvo-Turrubiartes MZ, Salgado-Bustamante M, Rodríguez-Pinal CY, Gámez-López LR, et al. Regulatory T Cells in children with intestinal parasite infection. Parasite Immunol. 2009 Oct;31(10):597–603.

17. Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, Maizels RM. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. J Immunol Baltim Md 1950. 2005 Apr 15;174(8):4924–33.

18. Nacher M, Gay F, Singhasivanon P, Krudsood S, Treeprasertsuk S, Mazier D, et al. Ascaris lumbricoides infection is associated with protection from cerebral malaria. Parasite Immunol. 2000 Mar;22(3):107–13.

19. LYKE KE, DICKO A, DABO A, SANGARE L, KONE A, COULIBALY D, et al. ASSOCIATION OF SCHISTOSOMA HAEMATOBIUM INFECTION WITH PROTECTION AGAINST ACUTE PLASMODIUM FALCIPARUM MALARIA IN MALIAN CHILDREN. Am J Trop Med Hyg. 2005 Dec;73(6):1124–30.

20. Lemaitre M, Watier L, Briand V, Garcia A, Le Hesran JY, Cot M. Coinfection with Plasmodium falciparum and Schistosoma haematobium: additional evidence of the protective effect of Schistosomiasis on malaria in Senegalese children. Am J Trop Med Hyg. 2014 Feb;90(2):329–34.

21. Sangweme DT, Midzi N, Zinyowera-Mutapuri S, Mduluza T, Diener-West M, Kumar N. Impact of Schistosome Infection on Plasmodium falciparum Malariometric Indices and Immune Correlates in School Age Children in Burma Valley, Zimbabwe. PLoS Negl Trop Dis. 2010 Nov 9;4(11):e882.

22. Sokhna C, Le Hesran J-Y, Mbaye PA, Akiana J, Camara P, Diop M, et al. Increase of malaria attacks among children presenting concomitant infection by Schistosoma mansoni in Senegal. Malar J. 2004 Nov 15;3:43.

23. Mulu A, Legesse M, Erko B, Belyhun Y, Nugussie D, Shimelis T, et al. Epidemiological and clinical correlates of malaria-helminth co-infections in Southern Ethiopia. Malar J. 2013;12:227.

24. Dolo H, Coulibaly YI, Dembele B, Konate S, Coulibaly SY, Doumbia SS, et al. Filariasis attenuates anemia and

proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults. PLoS Negl Trop Dis. 2012;6(11):e1890.

25. Noone C, Parkinson M, Dowling DJ, Aldridge A, Kirwan P, Molloy SF, et al. Plasma cytokines, chemokines and cellular immune responses in pre-school Nigerian children infected with Plasmodium falciparum. Malar J. 2013;12:5.

26. Lyke KE, Dabo A, Sangare L, Arama C, Daou M, Diarra I, et al. Effects of concomitant Schistosoma haematobium infection on the serum cytokine levels elicited by acute Plasmodium falciparum malaria infection in Malian children. Infect Immun. 2006 Oct;74(10):5718–24.

27. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly YI, Diallo AA, et al. Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol Baltim Md 1950. 2011 Apr 15;186(8):4725–33.

28. Panda M, Sahoo PK, Mohapatra AD, Dutta S kanti, Thatoi PK, Tripathy R, et al. Decreased prevalence of sepsis but not mild or severe P. falciparum malaria is associated with pre-existing filarial infection. Parasit Vectors. 2013;6:203.

29. Nacher M. Interactions between worms and malaria: good worms or bad worms? Malar J. 2011;10:259.

30. Harris N, Gause WC. B cell function in the immune response to helminths. Trends Immunol. 2011 Feb;32(2):80–8.

31. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. - PubMed - NCBI [Internet]. [cited 2015 Dec 22]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18625747/

32. Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. - PubMed - NCBI [Internet].

[cited 2015 Dec 22]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19592645/

33. Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. - PubMed - NCBI [Internet]. [cited 2015 Dec 22]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/?term=Malaria-associated+atypical+memory+B+cells+exhibit+markedly+reduced+B+cell+receptor+signaling+and+effector+function

34. Nacher M, Singhasivanon P, Silachamroon U, Treeprasertsu S, Krudsood S, Gay F, et al. Association of helminth infections with increased gametocyte carriage during mild falciparum malaria in Thailand. Am J Trop Med Hyg. 2001 Nov;65(5):644–7.

35. Talman AM, Domarle O, McKenzie FE, Ariey F, Robert V. Gametocytogenesis : the puberty of Plasmodium falciparum. Malar J. 2004 Jul 14;3(1):24.

36. van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JAM, Dodemont HJ, et al. A Central Role for P48/45 in Malaria Parasite Male Gamete Fertility. Cell. 2001 Jan 12;104(1):153–64.

37. Healer J, McGuinness D, Hopcroft P, Haley S, Carter R, Riley E. Complement-mediated lysis of Plasmodium falciparum gametes by malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs230. Infect Immun. 1997 Aug;65(8):3017–23.

38. Bousema T, Roeffen W, Meijerink H, Mwerinde H, Mwakalinga S, van Gemert G-J, et al. The dynamics of naturally acquired immune responses to Plasmodium falciparum sexual stage antigens Pfs230 & Pfs48/45 in a low endemic area in Tanzania. PloS One. 2010;5(11):e14114.

39. Ouédraogo AL, de Vlas SJ, Nébié I, Ilboudo-Sanogo E, Bousema JT, Ouattara AS, et al. Seasonal patterns of Plasmodium falciparum gametocyte prevalence and density in a rural population of Burkina Faso. Acta Trop. 2008 Jan;105(1):28–34.

40. Nacher M, Singhasivanon P, Yimsamran S, Manibunyong W, Thanyavanich N, Wuthisen R, et al. Intestinal helminth infections are associated with increased incidence of Plasmodium falciparum malaria in Thailand. J Parasitol. 2002 Feb;88(1):55–8.

41. Degarege A, Legesse M, Medhin G, Animut A, Erko B. Malaria and related outcomes in patients with intestinal helminths: a cross-sectional study. BMC Infect Dis. 2012;12:291.

42. Lyke KE, Dicko A, Dabo A, Sangare L, Kone A, Coulibaly D, et al. Association of Schistosoma haematobium infection with protection against acute Plasmodium falciparum malaria in Malian children. Am J Trop Med Hyg. 2005 Dec;73(6):1124–30.

43. Lemaitre M, Watier L, Briand V, Garcia A, Le Hesran JY, Cot M. Coinfection with Plasmodium falciparum and Schistosoma haematobium: additional evidence of the protective effect of Schistosomiasis on malaria in Senegalese children. Am J Trop Med Hyg. 2014 Feb;90(2):329–34.

44. Courtin D, Djilali-Saïah A, Milet J, Soulard V, Gaye O, Migot-Nabias F, et al. Schistosoma haematobium infection affects Plasmodium falciparum-specific IgG responses associated with protection against malaria. Parasite Immunol. 2011 Feb;33(2):124–31.

45. Lyke KE, Wang A, Dabo A, Arama C, Daou M, Diarra I, et al. Antigen-Specific B Memory Cell Responses to Plasmodium falciparum Malaria Antigens and Schistosoma haematobium Antigens in Co-Infected Malian Children. PLoS ONE [Internet]. 2012 Jun 5 [cited 2013 Jan 17];7(6). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3367916/

46. Inoue S-I, Niikura M, Mineo S, Kobayashi F. Roles of IFN-γ and γδ T Cells in Protective Immunity Against Blood-Stage Malaria. Front Immunol. 2013;4:258.

47. Clark IA, Budd AC, Alleva LM, Cowden WB. Human malarial disease: a consequence of inflammatory cytokine release. Malar J. 2006;5:85.

48. Gandhapudi SK, Tan C, Marino JH, Taylor AA, Pack CC, Gaikwad J, et al. IL-18 acts in synergy with IL-7 to promote ex vivo expansion of T lymphoid progenitor cells. J Immunol Baltim Md 1950. 2015 Apr 15;194(8):3820–8.

49. Amara S, Lopez K, Banan B, Brown S-K, Whalen M, Myles E, et al. Synergistic effect of pro-inflammatory TNFα and IL-17 in periostin mediated collagen deposition: potential role in liver fibrosis. Mol Immunol. 2015 Mar;64(1):26–35.

50. Keller CC, Yamo O, Ouma C, Ong'echa JM, Ounah D, Hittner JB, et al. Acquisition of hemozoin by monocytes down-regulates interleukin-12 p40 (IL-12p40) transcripts and circulating IL-12p70 through an IL-10-dependent mechanism: in vivo and in vitro findings in severe malarial anemia. Infect Immun. 2006 Sep;74(9):5249–60.

51. Ngoa UA, Zinsou JF, Kassa RFK, Feugap EN, Honkpehedji YJ, Massinga-Loembe M, et al. Assessment of the effect of Schistosoma haematobium co infection on malaria parasites and immune responses in rural populations in Gabon: study protocol. SpringerPlus. 2014 Jul 29;3(1):388.

52. Hürlimann E, Yapi RB, Houngbedji CA, Schmidlin T, Kouadio BA, Silué KD, et al. The epidemiology of polyparasitism and implications for morbidity in two rural communities of Côte d'Ivoire. Parasit Vectors. 2014;7:81.

53. Midzi N, Sangweme D, Zinyowera S, Mapingure MP, Brouwer KC, Munatsi A, et al. The burden of polyparasitism among primary schoolchildren in rural and farming areas in Zimbabwe. Trans R Soc Trop Med Hyg. 2008 Oct;102(10):1039–45.

54. Metenou S, Dembélé B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI, et al. Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population. J Immunol Baltim Md 1950. 2009 Jul 15;183(2):916–24.

55. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly YI, Diallo AA, et al. Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol Baltim Md 1950. 2011 Apr 15;186(8):4725–33.

56. Hartgers FC, Obeng BB, Kruize YCM, Dijkhuis A, McCall M, Sauerwein RW, et al. Responses to malarial antigens are altered in helminth-infected children. J Infect Dis. 2009 May 15;199(10):1528–35.

57. Wilson S, Jones FM, Mwatha JK, Kimani G, Booth M, Kariuki HC, et al. Hepatosplenomegaly associated with chronic malaria exposure: evidence for a pro-inflammatory mechanism exacerbated by schistosomiasis. Parasite Immunol. 2009 Feb;31(2):64–71.

58. Nmorsi O, Isaac C, Ukwandu N, Ekundayo A, Ekozien M. Schistosoma haematobium and Plasmodium falciparum co-infection with protection against Plasmodium falciparum malaria in Nigerian children. Asian Pacific journal of tropical medicine. :16–20.

59. Hartgers FC, Obeng BB, Kruize YCM, Dijkhuis A, McCall M, Sauerwein RW, et al. Responses to malarial antigens are altered in helminth-infected children. J Infect Dis. 2009 May 15;199(10):1528–35.

60. Hartgers FC, Obeng BB, Boakye D, Yazdanbakhsh M. Immune responses during helminth-malaria co-infection: a pilot study in Ghanaian school children. Parasitology. 2008 Jun;135(7):855–60.

61. Metenou S, Dembélé B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI, et al. Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population. J Immunol Baltim Md 1950. 2009 Jul 15;183(2):916–24.

62. Diallo TO, Remoue F, Gaayeb L, Schacht A-M, Charrier N, De Clerck D, et al. Schistosomiasis coinfection in children influences

acquired immune response against Plasmodium falciparum malaria antigens. PloS One. 2010;5(9):e12764.

63. Lyke KE, Wang A, Dabo A, Arama C, Daou M, Diarra I, et al. Antigen-specific B memory cell responses to Plasmodium falciparum malaria antigens and Schistosoma haematobium antigens in co-infected Malian children. PloS One. 2012;7(6):e37868.

64. Boef AGC, May L, van Bodegom D, van Lieshout L, Verweij JJ, Maier AB, et al. Parasitic infections and immune function: effect of helminth infections in a malaria endemic area. Immunobiology. 2013 May;218(5):706–11.

65. Schwartz L, Brown GV, Genton B, Moorthy VS. A review of malaria vaccine clinical projects based on the WHO rainbow table. Malar J. 2012 Jan 9;11:11.

66. RTS,S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet. 2015 Apr 23;

67. Esen M, Mordmüller B, de Salazar PM, Adegnika AA, Agnandji ST, Schaumburg F, et al. Reduced antibody responses against Plasmodium falciparum vaccine candidate antigens in the presence of Trichuris trichiura. Vaccine. 2012 Dec 14;30(52):7621–4.

68. von Hertzen L, Hanski I, Haahtela T. Natural immunity. Biodiversity loss and inflammatory diseases are two global megatrends that might be related. EMBO Rep. 2011 Nov;12(11):1089–93.

69. Mbow M, de Jong SE, Meurs L, Mboup S, Dieye TN, Polman K, et al. Changes in immunological profile as a function of urbanization and lifestyle. Immunology. 2014 Dec;143(4):569–77.

70. Smolen KK, Ruck CE, Fortuno ES, Ho K, Dimitriu P, Mohn WW, et al. Pattern recognition receptor-mediated cytokine response in

infants across 4 continents. J Allergy Clin Immunol. 2014 Mar;133(3):818–26.e4.

71. Labuda LA, de Jong SE, Meurs L, Amoah AS, Mbow M, Ateba-Ngoa U, et al. Differences in innate cytokine responses between European and African children. PloS One. 2014;9(4):e95241.

72. Yilmaz B, Portugal S, Tran TM, Gozzelino R, Ramos S, Gomes J, et al. Gut microbiota elicits a protective immune response against malaria transmission. Cell. 2014 Dec 4;159(6):1277–89.

73. Immunogenicity of influenza virus vaccine is increased by anti-gal-mediated targeting to antigen-presenting cells. - PubMed - NCBI [Internet]. [cited 2015 Dec 23]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17609270

74. Giacomin P, Zakrzewski M, Croese J, Su X, Sotillo J, McCann L, et al. Experimental hookworm infection and escalating gluten challenges are associated with increased microbial richness in celiac subjects. Sci Rep. 2015;5:13797.

Addendum

Nederlandse samenvatting

Eerdere onderzoeken, hoewel weinig in aantal, wijzen op een effect van helminthen (parasitaire wormen) op malariaziekteverschijnselen en op de afweerreactie tegen *Plasmodium spp* in gecoïnfecteerde personen. Echter zoals besproken in het eerste hoofdstuk van dit proefschrift, is het nog onduidelijk hoe consistent dit effect is tussen verschillende studiegebieden en wat de immunologische basis ervan is. Het onderzoek dat hier gepresenteerd wordt is er op gericht om het effect van helminthen op de afweerreactie tegen de malaria parasiet *Plasmodium* beter te begrijpen in gecoïnfecteerde personen in endemische landen. Dit proefschrift bevat data van studies die zijn verricht in landelijke en semi-stedelijke gebieden in Lambaréné (Gabon), waar malaria en helminthen veelvuldig voorkomen. We kozen voor een multidisciplinaire aanpak, zoals aangegeven in hoofdsuk twee, om de onderzoeksvragen te beantwoorden.

Antilichamen zijn van bijzonder belang gebleken voor de afweer tegen *Plasmodium spp*. en kunnen worden beïnvloed door helminthen. In hoofdstuk 3 hebben we een longitudinale studie opgezet om te bepalen in hoeverre *S. haematobium* infectie het fenotype en functie van B-cellen in chronisch geïnfecteerde kinderen beïnvloedt. Ten tweede hebben we de vraag behandeld of de verwijdering van de *S. haematobium*-parasiet met Praziquantel-behandeling de geobserveerde veranderingen kon herstellen. We vonden dat de verdeling van B-cellen anders was in *S. haematobium* geïnfecteerde personen, aangezien zij een hogere frequentie van geheugen-B-cellen hadden, tezamen met een verlaagde frequentie van naïeve B-cellen, in vergelijking met ongeïnfecteerde controle personen. Deze verandering door schistosomen omvatte ook de functie van B-cellen: de cellen waren meer geactiveerd na stimulatie, maar prolifereerden minder en produceerden minder TNF-α. Het is interessant om op te merken dat deze veranderingen gedeeltelijk hersteld waren na Praziquantel-behandeling. Hoewel we hier niet konden nagaan wat het effect was

van de door schistosome veroorzaakte veranderingen in B-cel functie en fenotype op anti-malaria-afweer, zijn er enkele studies die de hypothese onderschrijven dat *Plasmodium*-specifieke humorale afweer verzwakt kan zijn door helminthen. We hebben besloten deze vraag te beantwoorden in een epidemiologische studie beschreven in hoofdstuk 4. Deze studie was cross-sectioneel en bestond uit 287 personen van 6 tot 50 jaar oud. Deze individuen waren óf geïnfecteerd met *Plasmodium falciparum* alleen óf gecoïnfecteerd met *Plasmodium falciparum* en *Schistosoma haematobium*, *Loa loa* of *Mansonelle perstans*. Totaal IgG tegen seksuele en aseksuele stadia van *P. falciparum* is gemeten en vergeleken tussen beide groepen. Het belangrijkste resultaat dat we beschrijven was een significante afname van antilichamen tegen het *P. falciparum* seksuele stadium antigen *Pfs*48/45 in malaria- en schistosome-geïnfecteerde personen (maar niet met *L loa* of *M. perstans*). De door schistosoma veroorzaakte imuunmodulatie reikte niet tot Pfs230, een ander *P. falciparum* antigen van het seksuele stadium, of tot andere antigenen van aseksuele-stadia die waren getest. Dit is de eerste keer dat het effect van helminthen op de humorale afweerreactie tegen antigenen van seksuele stadia van *P. falciparium* zijn onderzocht. Onze bevindingen roepen vragen op over het effect van helminthen op malariaoverdracht, wat een nieuw onderzoeksveld behelst.

In gebieden waar veel helminth infecties voorkomen, begint de blootstelling aan helminthen al in de baarmoeder. Er is bewijs dat er transplacentaire overdracht plaatsvindt van filaria parasieten en antigenen van geïnfecteerde moeders naar de foetus. Bovendien is er aangetoond dat de nakomelingen van moeders die een helminthen infectie hadden tijdens de zwangerschap, een hoger risico lopen om later in hun leven geïnfecteerd te worden met deze parasieten. Uit immuno-epidemiologische studies is gebleken dat de immuuncellen van pasgeborenen afkomstig van moeders die geïnfecteerd zijn met filaria, minder sterk reageren op filaria antigenen in *in-vitro* experimenten. Het mechanisme van deze verminderde afweerreactie is

nog niet volledig bekend. Het is echter bekend dat de tolerantie voor helminthen in chronisch geïnfecteerde personen voornamelijk veroorzaakt wordt door de inductie van een krachtig netwerk van regulatoire cellen. Het is echter nog niet eerder onderzocht of de activatie van deze regulatoire cellen al in de baarmoeder plaatsvindt. Hoofdstuk 5 richt zich op dit vraagstuk. Voor deze studie, verricht in Lambaréné (Gabon), zijn dertig zwangere vrouwen onderzocht waarvan de helft was geïnfecteerd met filariaparasieten. We hebben de frequenties van de verschillende $CD4^+$ T cel subpopulaties gemeten in de immuun cellendie werden geïsoleerd uit de navelstreng van de pasgeborenen. Daarnaast is er onderzocht of er een relatie is tussen Th1 of Th17 cel subpopulaties en de regulatoire T cellen. We tonen aan dat de frequenties van $CD4^+$ T cel subpopulaties gelijk is in pasgeborenen van filaria-geïnfecteerde en ongeïnfecteerde moeders. Echter, in de pasgeborenen van filaria-geïnfecteerde moeders bleek er een negatieve correlatie te zijn tussen regulatoire T cellen en zowel de Th1 als Th17 $CD4^+$ T cel subpopulaties. Dit wijst erop dat er activering van regulatoire T cellen in de baarmoeder optreedt in moeders met een helmintheninfectie en dat deze cellen in staat zijn om cellulaire afweerreacties te onderdrukken.

Nader onderzoek naar het effect van helminthen op de cellulaire afweerreacties tegen *Plasmodium spp.* werd verricht in de studie beschreven in hoofdstuk 6. Deze studie biedt informatie over de aangeboren en verworven afweerreacties in schoolkinderen die geïnfecteerd zijn met *P. falciparum*, met of zonder gelijktijdige *S. haematobium* infectie. In plaats van de individuele cytokines te analyseren, zoals vaak wordt beschreven in immuno-epidemiologische studies, hebben we gekozen voor een meer globale aanpak om het patroon van cytokine responsen te onderzoeken. We hebben gevonden dat een *P. falciparum* infectie leidt tot een toename in aangeboren en verworven afweerreacties, terwijl personen met een *S. haematobium* infectie een toename in chemokines laten zien en een afname in pro-inflammatoire markers. Er was echter geen effect van de *S.*

haematobium infectie op de afweerreactie van kinderen met een *P. falciparum* infectie, dit geldt zowel voor de aangeboren aspecifeke als de verworven specifieke component van het afweersysteem. Hoewel deze studie een mogelijk effect van *S. haematobium* op de afweerreactie van *P. falciparum*-geïnfecteerde kinderen uitsluit, is het van belang om te erkennen dat de huidige studies rondom dit onderwerp tot nu toe tegengestelde resultaten hebben opgeleverd.

In hoofdstuk 7 hebben we een meta-analyse uitgevoerd waarin we alle studies samenvatten die het effect van helminthen op *plasmodium spp*. gerelateerde afweerreacties hebben onderzocht, en we hebben geanalyseerd welke factoren verantwoordelijk zijn voor de uiteenlopende resultaten. We tonen aan dat wanneer we alle studies samenvoegen, geen enkele onderzochte immuun parameter beïnvloed wordt door helminthen. Meer gespecialiseerde analyses laten echter zien dat dit resultaat verklaard kan worden door verschillende helminth soorten, eerdere blootstelling aan helminthen, het gelijktijdig voorkomen van meerdere helminth soorten en de immunologische analyses die zijn gedaan. Deze meta-analyse toont het belang aan van gestandaardiseerde studie ontwerpen en protocollen in immuno-epidemiologische studies.

Dit proefschrift heeft meer inzicht gegeven in de complexe interactie tussen helminthen en malaria in de menselijke gastheer en deze bevindingen wordt samengevat in hoofdstuk 8. Daarnaast beschrijven we in dit hoofdstuk hoe deze interactie in de toekomst verder onderzocht kan worden.

List of publications

1. Ateba Ngoa U, Jones S, Zinsou J, Honkpehedji YJ, Adegnika AA, Dejon Agobe JC, et al. Associations between helminth infections, Plasmodium falciparum parasite carriage and antibody responses to sexual and asexual stage malaria antigens. Am. J. Trop. Med. and Hyg. 2016 in press

2. Ateba-Ngoa U, Adegnika AA, Zinsou JF, Kassa Kassa RF, Smits H, Massinga-Loembe M, et al. Cytokine and chemokine profile of the innate and adaptive immune response of schistosoma haematobium and plasmodium falciparum single and co-infected school-aged children from an endemic area of Lambaréné, Gabon. Malar J. 2015;14:94.

3. Ateba-Ngoa U, Mombo-Ngoma G, Zettlmeissl E, van der Vlugt LEPM, de Jong SE, de Jong S, et al. CD4+CD25hiFOXP3+ cells in cord blood of neonates born from filaria infected mother are negatively associated with CD4+Tbet+ and CD4+RORγt+ T cells. PloS One. 2014;9(12):e114630.

4. Ateba Ngoa U, Zinsou JF, Kassa RFK, Ngoune Feugap E, Honkpehedji YJ, Massinga-Loembe M, et al. Assessment of the effect of Schistosoma haematobium co infection on malaria parasites and immune responses in rural populations in Gabon: study protocol. SpringerPlus. 2014;3:388.

5. Kenguele HM, Adegnika AA, Nkoma A-M, Ateba-Ngoa U, Mbong M, Zinsou J, et al. Impact of short-time urine freezing on the sensitivity of an established Schistosoma real-time PCR assay. Am J Trop Med Hyg. 2014 Jun;90(6):1153.

6. Adegnika AA, Zinsou JF, Issifou S, Ateba-Ngoa U, Kassa RF, Feugap EN, et al. Randomized, controlled, assessor-blind clinical trial to assess the efficacy of single- versus repeated-dose albendazole to treat ascaris lumbricoides, trichuris

trichiura, and hookworm infection. Antimicrob Agents Chemother. 2014 May;58(5):2535.

7. Labuda LA, de Jong SE, Meurs L, Amoah AS, Mbow M, Ateba-Ngoa U, et al. Differences in innate cytokine responses between European and African children. PloS One. 2014;9(4):e95241.

8. Scherbaum M, Kösters K, Mürbeth RE, Ngoa UA, Kremsner PG, Lell B, et al. Incidence, pathogens and resistance patterns of nosocomial infections at a rural hospital in Gabon. BMC Infect Dis. 2014;14(1):1.

9. Jepsen MPG, Jogdand PS, Singh SK, Esen M, Christiansen M, Issifou S, et al. The malaria vaccine candidate GMZ2 elicits functional antibodies in individuals from malaria endemic and non-endemic areas. J Infect Dis. 2013 Aug 1;208(3):479.

10. Labuda LA, Ateba-Ngoa U, Feugap EN, Heeringa JJ, van der Vlugt LEPM, Pires RBA, et al. Alterations in Peripheral Blood B Cell Subsets and Dynamics of B Cell Responses during Human Schistosomiasis. PLoS Negl Trop Dis. 2013 Mar 7;7(3):e2094.

11. Esen M, Mordmüller B, de Salazar PM, Adegnika AA, Agnandji ST, Schaumburg F, et al. Reduced antibody responses against Plasmodium falciparum vaccine candidate antigens in the presence of Trichuris trichiura. Vaccine. 2012 Dec 14;30(52):7621.

12. Ateba Ngoa U, Schaumburg F, Adegnika AA, Kösters K, Möller T, Fernandes JF, et al. Epidemiology and population structure of Staphylococcus aureus in various population groups from a rural and semi urban area in Gabon, Central Africa. Acta Trop. 2012 Oct;124(1):42.

13. Ngoa UA, Adzoda GK, Louis BM, Adegnika AA, Lell B. Buruli ulcer in Gabon, 2001-2010. Emerg Infect Dis. 2012 Jul;18(7):1206.

14. van der Vlugt LEPM, Labuda LA, Ozir-Fazalalikhan A, Lievers E, Gloudemans AK, Liu K-Y, et al. Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. PloS One. 2012;7(2):e30883.

15. Bélard S, Issifou S, Hounkpatin AB, Schaumburg F, Ngoa UA, Esen M, et al. A randomized controlled phase Ib trial of the malaria vaccine candidate GMZ2 in African children. PloS One. 2011;6(7):e22525.

16. Schaumburg F, Ngoa UA, Kösters K, Köck R, Adegnika AA, Kremsner PG, et al. Virulence factors and genotypes of Staphylococcus aureus from infection and carriage in Gabon. Clin Microbiol Infect Dis. 2011 Oct;17(10):1507.

17. Meurs L, Labuda L, Amoah AS, Mbow M, Ngoa UA, Boakye DA, et al. Enhanced pro-inflammatory cytokine responses following Toll-like-receptor ligation in Schistosoma haematobium-infected schoolchildren from rural Gabon. PloS One. 2011;6(9):e24393.

18. Schaumburg F, Köck R, Friedrich AW, Soulanoudjingar S, Ngoa UA, von Eiff C, et al. Population structure of Staphylococcus aureus from remote African Babongo Pygmies. PLoS Negl Trop Dis. 2011;5(5):e1150.

19. Adegnika AA, Ramharter M, Agnandji ST, Ateba Ngoa U, Issifou S, Yazdanbahksh M, et al. Epidemiology of parasitic co-infections during pregnancy in Lambaréné, Gabon. Trop Med Int Health TM IH. 2010 Oct;15(10):1204.

20. Koko J, Ategbo S, Ateba Ngoa U, Moussavou A. Étude épidémiologique de la carie dentaire en milieu scolaire à Libreville, Gabon. Clin Mother Child Health 2009; 6: 1065.

Curriculum vitae

Ulysse Ateba Ngoa was born in Cameroon in March 1980. After his primary and secondary school in Senegal, Cameroon and Gabon he was admitted to the Faculty of Medicine of Libreville (Gabon) in 1998. He obtained his medical degree in 2008 and joined the Centre de Recherches Médicales de Lambaréné at the Albert Schweitzer Hospital. There he started working as a clinical investigator and was first involved in projects aiming at dissecting the effect of helminths and particularly schistosomiasis on the immune responses of infected individuals living in rural and semi-urban areas of Lambaréné. His research interest extended to the epidemiology of Staphylococcus aureus carriage, Buruli ulcer in Gabon as well as to vaccine trials on malaria. In 2011 he was enrolled in a PhD program at the Leiden University under the supervision of Professor Maria Yazdanbakhsh. His PhD work focused on host parasite interaction particularly assessing the effect of helminths on *Plasmodium falciparum* induced immune responses in co-infected individuals living in an endemic area in Gabon. In 2015 while finalizing his thesis, Ulysse Ateba Ngoa resumed his work at the CERMEL where he was appointed as a principal investigator in a phase I/IIa trial to study the efficacy of GMZ2-CAF01, a blood stage malaria vaccine candidate. To assess this he conducted a controlled human malaria infection model in Gabonese adults. He has been awarded a three year funding from the DFG, as a co-applicant, to assess the effect of helminths on malaria transmission.

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