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General Discussion.

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

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

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

Innate Immune Responses

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

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

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

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

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

Adaptive Immune Responses

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

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

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

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

Regulatory Responses

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

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

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

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

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

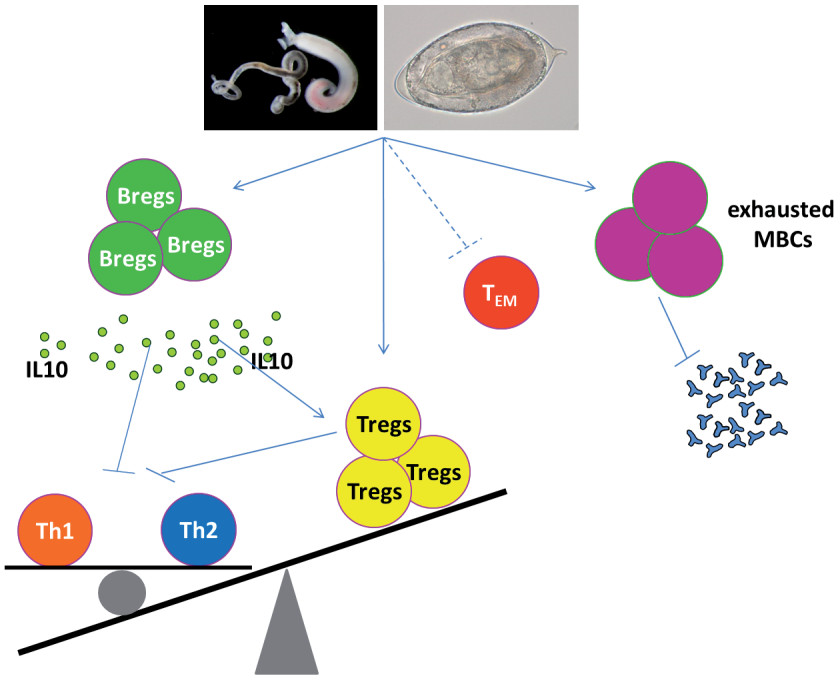

Figure 1. A schematic representation of innate, adaptive and regulatory immune responses in human schistosomiasis in Gabon.

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

Immunosupression as a result of anergy

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

Spill-over suppression

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

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

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

Concluding remarks and future perspectives

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

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

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

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

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

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

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