CHAPTER 9

Summarizing Discussion

Future directions

While the poorest areas in the world suffer from a high burden of helminth infections, the same parasites are currently proposed to be beneficial in hampering inflammatory diseases in the richest areas of the world. The immune regulatory network induced by helminths may explain both sides of the coin; the chronic presence of worms in the human host and the dampening of pathological inflammatory conditions.

This thesis – in the context of the ImmunoSPIN project (www.immunospin.org) – has contributed to further understanding of immune modulation exerted by parasites. Regarding the aims of this thesis, the main findings were that (i) polarized T-helper cell and Treg subsets can be detected in peripheral blood of parasitized patients, (ii) Tregs functionally contribute to suppression of parasite-specific and bystander responses, (iii) deworming restores helminth-induced general and parasite-specific immune responsiveness and (iv) these immunological observations do not immediately lead to evident clinical consequences, leaving the question whether they may elicit relevant effects in the longer term, unanswered.

Characterization of T-helper & Tregs during parasitic infection

The thesis starts with the characterization of Tregs as well as T-helper subsets during parasitic infections and the role that Tregs have in controlling immune responses. In **chapter 2** we show that the proportion of $CD4^+CD25^{hi}FOXP3^+$ Tregs is higher in individuals with detectable *Loa loa* microfilaria (MF) compared to uninfected endemic controls. Interestingly, the Treg population is also increased in the group with no patent infection, yet positive for filaria-specific IgG4 and with a recent history of eye worm passage, which indicate that these subjects are infected but amicrofilaremic. Both infected groups displayed lower Th1 and Th17 responses to filarial antigens compared to the control group. Interestingly, in the infected but amicrofilaremic group, higher Th2 and IL-10 responses were measured compared to MF-positives and endemic controls. This suggests that immune regulatory mechanisms are induced during different stages of *Loa loa* infection, but are possibly more profound in individuals carrying MF. When we assessed Treg phenotype in asymptomatic malaria infections (**chapter 3**), the $CD4^+CD25^{hi}FOXP3^+$ subset was not different in parasitemic individuals, however the TNFRII-expressing Tregs were significantly elevated in the infected, suggesting a role for this specific subtype of Tregs in malaria infection. Surprisingly, malaria-induced Th1 responses were not altered in the infected group, whereas Th2 responses were lower. These findings were confirmed after treatment of malaria-positive children, revealing a decrease in TNFRII expression by Tregs and an increase in malaria-specific IL-13 production, in parallel to an expansion in the $GATA3^+$ subset. These data are in line with the view that Tregs are involved in suppression of different T-helper responses. With respect to the malaria study in **chapter 3**, it is interesting that malarial parasites, similar to helminths, are also able to induce the expansion of Tregs, supported by earlier studies[1,2]. However, previous studies have not assessed Th2 cells in any great detail. The results of our study along with the observation that the Fulani, a West-African tribe that is more resistant to malaria infection, express higher levels of *IL4* and *GATA3* genes[3], suggest that Th2 responses might need to be considered as important players, worth investigating in malaria immunity.

The field of Treg characterization is rapidly expanding. Within a few years, assessment of Tregs in parasitic diseases developed from indirect immunofluorescence of PBMC[4], through measuring mRNA expression of regulatory molecules and cytokines in PBMC[5-8], to distinguishing cell subsets co-expressing Treg markers by flow cytometry[9-12]. Currently it has become possible to measure up to 17 cell markers at a single cell level by flow cytometry. Staining of surface molecules and intracellular cytokines can be combined to define functional T cell subsets[13,14], but also transcription factors and even proliferative responses can be assessed simultaneously[15]. The phenotypic definition of Tregs remains a matter of

debate. Even in the few studies on Tregs in human helminth infections, markers used to distinguish Tregs differ considerably (Table 1). Along the way there has been confusion on the discrimination of regulatory and activated T cells. CD25, component of the IL-2 receptor, was already known as an activation marker for T cells and more confusing was the observation that FOXP3 expression was also seen after activation of naive T cells, without associated suppressive activity[16]. Several other markers have been proposed to be specific for Tregs, among others, increased expression of cytotoxic T cell antigen (CTLA-)4[17], glucocorticoid receptor tumor necrosis factor receptor (GITR)[18,19], GARP[20], Helios[21] and absence of CD127[22,23]. However, the expression of all these markers appears to be largely dynamic and strictly speaking not Treg-specific[24]. The current view on identification of human Tregs is based on FOXP3, CD45RA and CD45RO, in which naive ($CD25^{high}FOXP3^{low}CD45RA^{+}CD45RO^{-}$) Tregs are distinguished from effector ($CD25^{high}FOXP3^{high}CD45RA^{-}CD45RO^{+}$) Tregs, both capable of suppression[25,26]. Activated effector T cells are then characterized by low FOXP3, low or high CD25 and CD69 expression in combination with IL-2 production[26]. Recently, it has been shown that the methylation of the *FOXP3* locus might be an important marker for Tregs with strong suppressive function. It has been proposed that the amount of FOXP3 demethylation can distinguish $FOXP3^{+}$ Tregs from $FOXP3^{+}$ activated conventional T cells, since only Tregs seem to display demethylation and activated T cells not[27].

Table 1. Overview of phenotypic definition of Tregs in human helminth studies by flow cytometry

Helminth species	Disease stage	Study population	Study site	Treg definition	Reference
Wuchereria bancrofti	microfilaremic, CA-positive	adults	India	$CD4^+IL\text{-}10^+$ *	No.5
Wuchereria bancrofti and/or *Mansonella perstans*	microfilaremic	adolescents	Mali	$CD4^+CD25^+FOXP3^+CD127^-$	No.14
				$CD4^+FOXP3^-IL\text{-}10^+$ **	
Schistosoma haematobium	chronic infection	children & adults	Zimbabwe	$CD4^+CD25^+FOXP3^+$	Nausch et al.
				$CD4^+CD25^{hi}$	*PLoS One 2011 (6):e16860*
				$CD4^+FOXP3^+CD127^-$	
Schistosoma mansoni	chronic infection	adults	Kenya	$CD4^+CD25^{hi}$	No.12
				$CD4^+CD25^{hi}CD45RO^+$	
Schistosoma mansoni	chronic infection	all ages	Brazil	$CD4^+CD25^{hi}$	Teixeira-Carvalho et al.
	chronic pathology			$CD4^+IL\text{-}10^+$ *	*Acta Trop 2008;108:139-49*
Schistosoma mansoni and/or *Plasmodium falciparum*	chronic infection	school children	Mali	$CD4^+CD25^{hi}FOXP3^+$	Lyke et al. *PLoS One 2012;7:e31647*
intestinal parasites and/or protozoa	chronic infection	school children	Mexico	$CD4^+CD25^{hi}$	No.9
				$CD4^+FOXP3^+$	
				$CD4^+CTLA\text{-}4^+$	
				$CD8^+CD28^-$	
geohelminths	chronic infection	school children	Indonesia	$CD4^+CD25^{hi}FOXP3^+$	Wammes et al. *Eur J Imm 2010;40:437-42*
Necator americanus	chronic infection		Brazil	$CD4^+CD25^+FOXP3^+$	No.11
Strongyloides stercoralis and/or HTLV	chronic infection	adults	Peru	$CD4^+CD25^{hi}FOXP3^+$	No.10
				$CD4^+CD25^{hi}$	

* *after PBMC stimulation with parasite antigens*
** *determined in unstimulated whole blood*

Moreover, the possibilities of cell sorting have allowed scientists to separate cell subsets based on surface markers, including positive selection for CD25 and negative selection for CD127[23] and CD49d[28], after which expression levels of other surface and/or intracellular components and functional capacities can be characterized more directly. The functional studies are based on suppression assays in which Tregs and T effector cells are co-cultured to evaluate inhibitory capacities of Tregs[29]. In translational research this has been extended to *in vitro* expansion of isolated Tregs for the purpose of immunotherapy[30].
However, when studying Tregs and T-cell immunology in parasite-endemic areas, experimental possibilities are limited. We, along with others, have shown that functional capacity of Tregs can nevertheless be studied in the field where facilities are restricted. By depleting $CD4^{+}CD25^{hi}$ cells from PBMC, freshly isolated from peripheral blood, it is possible to compare the proliferative and cytokine production capacity of PBMC with and without Tregs. This method has been used in chapters 4 and 5. In **chapter 4** we studied three clinical groups in an area endemic for lymphatic filariasis caused by *Wuchereria bancrofti*. We report suppressed Th1, Th2, Th17 and T-helper cell proliferative responses to filarial antigen in microfilaremic individuals compared to endemic controls. In contrast, subjects with chronic pathology displayed similar or enhanced Th1 and Th17 cytokine levels in comparison to the other two groups. After depletion of Tregs, proliferative and Th2 cytokine responses of MF-positives were restored to levels observed in endemic controls, indicating that indeed in microfilaremics, functional regulatory T cells exist that suppress some of the effector T cell responses. Whether the parasites induce Tregs to hide from the immune system or the host-generates Tregs in order to prevent the pathological consequences of infection, remains to be determined.

Immune correlates for infectious and inflammatory diseases

We were furthermore interested in the phenomenon of spill-over suppression. Downmodulation of responses to third party antigens by helminths could on the one hand lead to impaired responses to important infections[31,32] or vaccines[33], but on the other protect against excessive inflammatory responses observed in allergies, asthma and autoimmune diseases[34,35].
Chapter 5 presents the results of our study on bystander responses during geohelminth infections. Although the proportion of Tregs was not altered in infected children, Tregs from geohelminth-positive individuals displayed greater suppressive capacity deduced from the stronger enhancement of proliferation and IFN-γ secretion by PBMC in response to BCG vaccine and *P. falciparum*-infected red blood cells, after Treg depletion. Since this effect was not observed in helminth-

uninfected classmates, we concluded that Tregs with potent suppressory capacity are specifically triggered by the presence of helminths. The most elegant method of assessing the direct effect of helminths is to perform a randomized placebo-controlled study of helminth treatment. Few small-scale studies have previously shown that albendazole treatment increases effector T cell responses to BCG[36] and cholera[37] vaccination, and that it may also boost helminth-specific immune responses[38], although the latter study was not placebo-controlled. Two trials of the anti-schistosomal drug praziquantel have been carried out. A placebo-controlled study in pregnant women showed improved schistosome-specific cytokine production after treatment[39] and similarly, in an open-label trial, praziquantel administration to school children led to increased schistosomal antigen-induced IL-5 responses[40]. So far, no large-scale community anthelmintic treatment trials have been undertaken to assess the impact of deworming on immune responses and clinical outcomes. In **chapter 6** we have analyzed the effect of community-based deworming on cytokine responses to different stimuli. Three-monthly albendazole treatment only partly reduced the burden of STH infections in the community after 7 doses, however it strongly enhanced pro-inflammatory cytokine responses, especially to malaria antigens and mitogen. To ascertain that this is a helminth-specific and not a drug effect, we stratified the analysis based on helminth infection status, which revealed a prominent and in some cases stronger effect of treatment in the helminth-positive group. However, when interpreting these data, it should be noted that the number of helminth-uninfected individuals was relatively small. We also considered other possible confounders in this analysis. Helminths affect the nutritional status of an individual and malnutrition can be a cause of immune hyporesponsiveness[41]. We showed that body mass index did not change after treatment and moreover, helminth infection or albendazole treatment did not alter fasting blood glucose levels (unpublished data). This indicates that the alleviation of immune hyporesponsiveness is most likely due to the clearance of helminth infections, reducing their immune modulatory capacity. However, since our study was not designed to assess nutritional markers, we did not measure other, possibly more informative, parameters to fully exclude the possibility of improved nutritional status as a confounder.

In parallel we assessed the effect of deworming on several clinical outcomes: malaria prevalence and symptoms, SPT reactivity to allergens and symptoms of allergy, as presented in **chapter 7**. Malarial parasitemia was transiently increased in the treated group, although the prevalence of symptoms did not change. In contrast to our finding, earlier studies have indicated that helminth infection could lead to increased malaria parasitemia, but may protect from severe malarial disease[32]. An important limitation of our study was that during our trial, the prevalence of malaria decreased substantially, which might affect the results obtained. For allergic responses, there was an incremental increase in the

proportion of children with SPT reactivity to any of the allergens in the albendazole arm after 9 and 21 months, which did not reach statistical significance. However, when cockroach allergen was considered separately, a significant increase of SPT positivity was observed after treatment. It must be noted that deworming in this trial was not sufficient, suggesting that even more intensive anthelmintic treatment, possibly a combination of drugs, or drugs and environmental control, is needed for future studies. The fact that deworming was not complete also indicates that more profound changes in immune responses and clinical outcomes might be anticipated when more intensive deworming is achieved.

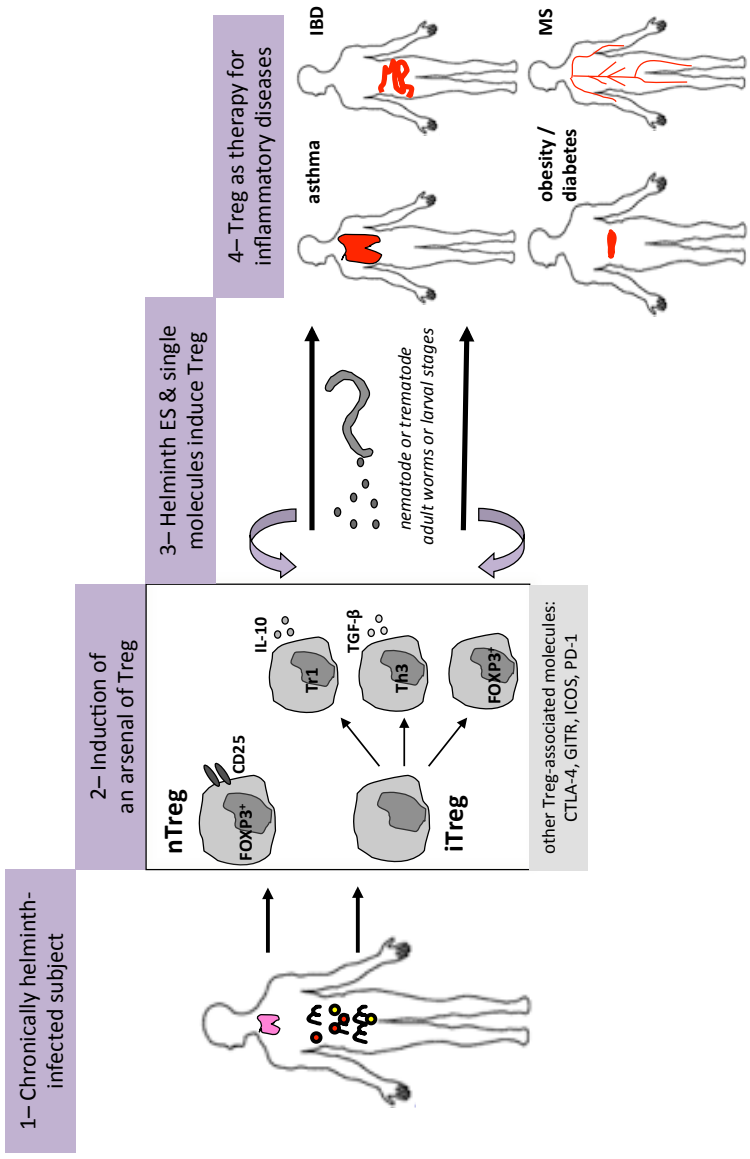
Comparing the results of **chapter 6** and **chapter 7**, we may conclude that *in vitro*-stimulated immune responses are not reflected in the clinical outcomes, at least not within our trial period. Clinically evident effects of deworming may take longer to develop, as illustrated by a study in Ecuador, which showed a major increase in SPT reactivity and possibly more eczema in communities that were enrolled in more than 15 years of regular treatment with ivermectin[42]. An earlier study by the same group showed that one year of two-monthly albendazole treatment of school children resulted in enhanced Th2 responses to helminth antigens and bacterial superantigen, but no change in allergen responses, SPT reactivity or allergic symptoms[38,43]. Although not in any great detail, immune responses have also been characterized in experimental helminth infections of humans, in the context of helminthic therapy for inflammatory diseases. Similarly, in these trials immunological responses did not always coincide with clinical findings. The report of an RCT of *Trichuris suis* ova (TSO) in allergic rhinitis patients showed increased Th2 and IL-10 responses to the introduced *Trichuris* infection[44], but no change in allergic outcomes were seen[45], supported by lack of any change in allergen-specific cytokine responses. This may imply that helminth-induced immunity may first be directed at helminth-specific responses, followed by modulation of bystander responses, leading to clinical effects at a later stage.

Future directions

First of all, there is no doubt that deworming campaigns should be further advocated[46]. However, it is of utmost importance to follow-up communities where mass drug administration (MDA) has taken place. Deworming may negatively affect coinfections[32], positively affect inflammatory diseases[42,47] and moreover the effect of anthelmintic treatment of pregnant women can become apparent in their offspring, leading to an increased prevalence of eczema[48]. However, some studies have provided evidence for beneficial effects of anthelmintic treatment, for example on malaria incidence[48] and CD4 counts in HIV infection[49]. Since the possibilities of performing placebo-controlled trials are complicated in some countries, further studies are needed that compare treated and untreated communities, to determine long-term effects of mass treatment. In areas where MDA is operational, coinfections and possible development of inflammatory conditions should be monitored while measurement of immunological markers might help understand causation.

At the same time, several clinical trials are running on the use of helminthic therapy to treat inflammatory diseases, as summarized in **chapter 8**. As treatment with full infections might not be the most ideal option, some research groups have been focusing on characterization of helminth-derived molecules that may be able to substitute the whole parasite approach[50]. With the current possibilities of preparing antigen mixtures or isolating single molecules from helminths, several products with immune modulating properties have been defined, however none has been administered to humans. Currently, the filarial product ES-62 is the best-characterized candidate molecule for therapeutic trials; it has shown promising data in murine models[51,52] and in a human *in vitro* model for arthritis[51]. Another product from filarial worms, AvCystatin, has also been shown to be protective for murine allergic airway inflammation[53] and moreover, *in vitro* it suppresses exaggerated Th2 responses of PBMC from grass pollen-allergic patients[54]. Excreted-secreted (ES) products of intestinal worms from *H. polygyrus* (HES) and ES from canine hookworm *Ankylostoma caninum*, have been shown to suppress allergic airway inflammation[55] and colitis[56] in mice, respectively. With regard to schistosomes, soluble worm extracts of *S. mansoni* have had beneficial effects against murine colitis[56], while a Lewis^{x}-containing glycan from *S. mansoni* eggs, lacto-N-fucopentaose III (LNFPIII), was able to suppress EAE[57] and psoriasis[58].

Taken together, these studies and results in animal models encourage the use of helminth-derived molecules, which might reproduce or even surpass the results of a full helminth infection. In particular, molecules associated with Tregs may be promising[59,60], since there is accumulating evidence that Tregs orchestrate the helminth-induced immune regulatory network (Figure 1).


1– Chronically helminth-infected subject
2– Induction of an arsenal of Treg
3– Helminth ES & single molecules induce Treg
4– Treg as therapy for inflammatory diseases
nTreg
CD25
FOXP3+
iTreg
IL-10
Tr1
TGF-β
Th3
FOXP3+
other Treg-associated molecules: CTLA-4, GITR, ICOS, PD-1
nematode or trematode adult worms or larval stages
IBD
asthma
MS
obesity / diabetes

Concluding remarks

Helminths are potent immune modulators and this can have both beneficial and detrimental consequences. Immune hyporesponsiveness is – at least partly – exerted by suppressive capacities of Tregs (**chapters 4 & 5**). Assessment of Treg phenotype and function is possible in areas with limited resources (**chapters 2 – 5**) and Tregs may have favorable properties in terms of dampening the excessive inflammation observed in allergies and autoimmune diseases. The immune regulatory network has gained significant attention as an important part of the web of immune responses, genetics and environmental factors that explain the current disease patterns seen upon epidemiological transition. Future clinical studies of deworming as well as helminthic therapy should encompass studies that will monitor different aspects of this regulatory network. Furthermore, larger and longer anthelmintic treatment RCTs might be needed to assess the immunological and clinical consequences of deworming. Given that there is some evidence on the possible detrimental effects of helminth elimination on the prevalence of allergies, asthma and other inflammatory diseases, it would be important to implement proper monitoring of MDA programs, specifically for altered immunological and clinical outcomes. Together, these strategies may help us to properly anticipate the double burden, of infectious and inflammatory diseases, in resource-poor settings.

Figure 1 (left page). Helminth-associated Tregs: lessons learnt and future directions. A schematic representation of the role of Tregs in infectious and inflammatory diseases. Helminths are associated with expansion of Tregs, which allows their long-term survival within their host (1). Tregs are comprised of both natural Tregs (nTregs), derived from the thymus, which are $FOXP3^+$ and express high levels of CD25, as well as different inducible (i)Tregs (2). So far, Tr1 and Th3 cells have been described, which do not always express FOXP3. Moreover, FOXP3 expression has also been seen in cells that do not express other Treg markers and are induced in the periphery. It should be noted that various markers such as CTLA-4, GITR, ICOS or PD-1 have been reported to be expressed on Tregs. Several helminth-derived compounds, such as excreted-secretory (ES) products and even single molecules, have been shown to either directly induce FOXP3 expression in T cells or condition dendritic cells to stimulate the expansion of Tregs (3). Although this is largely based on murine studies, it holds promise for future human studies. Ultimately, it may be possible to apply isolated Tregs clinically in a range of diseases (4), all characterized by high levels of inflammation. Future mechanistic studies may be needed to determine which Treg subsets are particularly suited for the different clinical conditions.

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