Tracking helminths : from molecular diagnostics to mechanisms behind immune polarization

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Chapter 8

General Discussion

Conceptual framework of this thesis

Knowing that helminth infections can have both detrimental and beneficial effects on host physiology and wellbeing, these consequences should be taken into account when one aims to implement programs to eradicate these infections. In this regard, decisions on when and where intervention strategies are initiated is on the one hand dependent on the availability of sensitive diagnostic methods to detect helminth infections, and on the other, on how these parasites influence the host immune system, directly or indirectly for instance via microbiota, as a major determining factor of disease outcome. Thus helminth diagnostics and immunological interplay between helminth and host can be seen as *two sides of the same coin* as they are distinct topics but also interrelated. The common denominator of the studies described in this thesis is tracking helminths, either, at the level of diagnostics or at the level of the immunological footprint they leave behind in the host. Together this thesis, provides valuable new insights for the design of more effective intervention strategies as well as for the development of therapeutic agents against inflammatory diseases. The conceptual frameworks of this thesis, is shown in the Figure 1. In this chapter, we will integrate the findings of different chapters. We discuss what novel insights this thesis has generated in the light of current knowledge and highlight directions for future research.

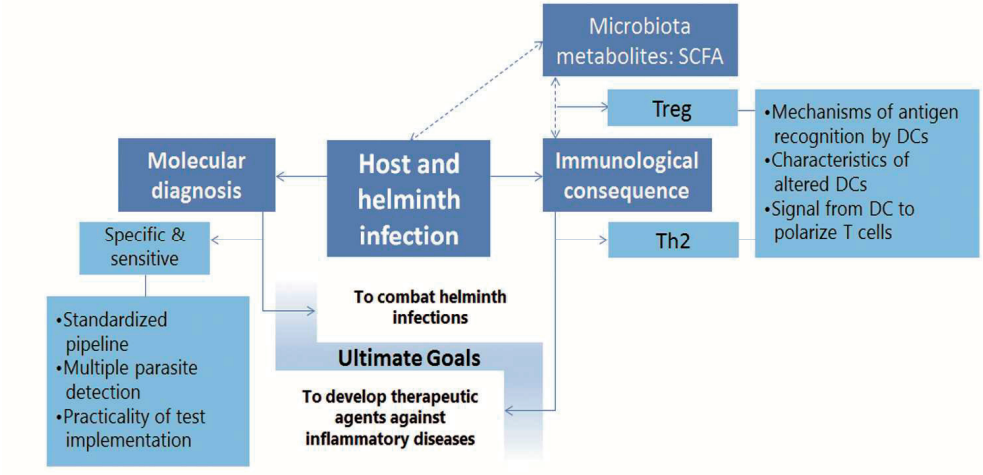

Figure 1. The conceptual framework of this thesis

Tracking helminths using molecular diagnostics, in a standerdized pipeline that allows for detection of multiple helminth species simulatioulsy with improved sensitivity and specificity over conventional methods, will be crucial for effective implementation and monitoring of deworming programs. The second part of this thesis focuses on tracking the immunological footprint of heliminths, particularly on the mechanisms of how helminths prime Th2 and Treg responses, either through direct functional modulation of DCs by helminth derived antigens, or indirectly by promoting changes in composition of gut microbiota and microbial products, such as SCFAs. Delineating the molecular mechanims through which helminths polarize Th2 and Treg response may now open up new avenues for anti-helminth therapies, but could also help in the developing of approaches to shape these immune responses for treatment of non-infectious inflammatory diseases.

Tracking helminths in the context of diagnostics

What we know and have learned from this thesis

Epidemiological studies have shown that the prevalence of infections caused by helminths, especially soil transmitted helminths (STHs) and schistosomes remain high in developing countries

and thus could have detrimental effects on the quality of life for humans [1, 2]. Although so far the diagnosis of helminth infections has relied on microscopy-based methods [3, 4], the importance of molecular based diagnostics has started to be appreciated. The lack of standardized protocols in performing microscopy-based methods to diagnose helminth infections [3, 5, 6], make it difficult to compare the data collected between studies [4, 7]. By evaluating the performance of multiplex real-time PCR, using samples collected in two helminths endemic areas, in which the microscopy procedures used were different, we have now shown that real-time PCR-based diagnosis can make the comparison between different endemic areas possible (**Chapter 2**). In addition, within a multiplex format, different parasite targets can be detected in a single procedure. We were also able to demonstrate that using real-time PCR two different species of hookworm namely *Necator americanus* and *Ancylostoma duodenale* can be distinguished, which is impossible by microscopy. This is important, because recently it was found that *A. duodenale,* but not *N. americanus*, is associated with severe anaemia and iron deficiency [8]. Moreover, *Strongyloides stercorales* can readily be detected by real-time PCR, whereas this is challenging using microscopy [9]. This may be particularly relevant, since it was recently shown that although 60% of infected people remain asymptomatic [10], immunosuppression in patients with chronic *S. stercorales* infection can precipitate a life-threatening hyper-infection characterized by increased parasite burden [11]. Finally, we have also demonstrated that the real-time PCR method is useful to analyse the risk factors associated with helminth infections. A recent study focusing on water, sanitation and hygiene (WASH) and environmental risk factors for infections with STHs, successfully used real-time PCR to identify strong risk associations with environmental variables [12].

Although real-time PCR-based methods to diagnose helminth infections have been reported to outperform microscopy-based methods [13-19], detection of *T. trichiura* by real-time PCR has often been unreliable until now because the current methods were insufficient to efficiently extract DNA from *T. trichiura* eggs. Therefore, specific sample preparation is required to extract the DNA of *T. trichiura* eggs prior to PCR detection. Bead-beating prior to DNA extraction has been used to improve DNA recovery. However, the studies using this method showed a PCR-based prevalence of *T. trichiura* below 3% [14, 17, 18, 20], which is a too low frequency to detect any beneficial effect that the beating method may have. Therefore, we performed in **Chapter 3** a comprehensive comparison of different sample preparation procedures prior to DNA extraction for helminth infections to establish a protocol to maximize *T. trichiura* DNA yield. We found that for optimal *T. trichiura* DNA extraction, sample preservation with ethanol in combination with bead-beating prior to parasite DNA extraction was the best procedure. This has already been successfully implemented in a recent population-based study where the effect of anthelmintic treatment on insulin resistance was examined [21].

Key points regarding current knowledge and the advances described in this thesis regarding the molecular diagnosis of helminth infections are summarized in Box.1, in section A and B, respectively.

Future perspective: A standardized operating procedure to perform epidemiological studies of helminth infections

The ability to reliably monitor the distribution of helminth infections is key to evaluate the success of mass drug administration programs [1, 2]. This critically depends on standardized diagnostic techniques for these infections and the implementation of strict guidelines of the operating procedures for diagnosis. We in chapter 2 and 3, not only indicate that diagnosis based on DNA detection is as a more reliable approach than microscopy, but also show that operating

procedures, which include sample collection, storage, preparation and helminth detection may affect the outcome of helminth diagnostics. However, it is well understood that each centre might have their own standard operating procedure in performing the diagnostics of helminth infections, raising the question, how can this be standardized? This will require, engagement of multiple research centres, the support from developed countries as well as consultation with WHO. Moreover, there is an emerging need for rapid diagnosis to support the WHO programs that strive to eliminate neglected tropical diseases (NTDs) in general and STHs and schistosomiasis, specifically. In this respect, recombinase polymerase amplification has recently been developed as an alternative molecular-based rapid diagnostic tool [22], that although still being evaluated in a population-based study, it is likely to become of great value for this field.
See for a summary of the direction for future research regarding molecular diagnostics of helminth infections Box.1, section C.

Tracking the immunological footprint of helminths in their host: Dendritic cell-driven T cell polarization by helminths

What we know and have learned from this thesis

Helminths induce Th2 polarization, in which DCs play a pivotal role. This is a complex process that is initiated by the recognition of antigens derived from helminths by DCs, via a variety of PRRs that in both signalling-dependent and independent mechanisms can condition DCs for Th2 polarization [23-27]. Yet the molecular mechanisms through which helminths instruct DCs to prime Th2 responses and how helminth-conditioned DCs drive Th2 polarization are still incompletely understood. In chapters 4, 5 and 6 we used various approaches to study this in detail by focusing on Th2 polarization by egg-derived antigens from *S. mansoni*.

In a first attempt to find markers important for Th2 induction by DCs we performed a proteomics analysis on DCs that were stimulated with SEA and ω-1 (**Chapter 5**). Although some groups have used proteomics to characterize DCs stimulated with helminth antigens, the studies were primarily gel-based [28-30]. However, gel-based methods do not allow for high-throughput analysis and direct comparison of biological replicates is not possible. In our study, we analysed the proteomes using LC-FTICRMS, a high-throughput gel-free technique based on accurate mass tag (AMT) analysis [31, 32]. This method enabled us to take into account donor-to-donor variation. We observed that DCs stimulated with SEA or ω-1 were clustered together and separated from IFN-γ-stimulated pro-Th1 DCs, indicative of distinct proteomics profiles in pro-Th1 *versus* pro-Th2 DCs. We found that both SEA and ω-1 strongly increased expression of 60S acidic ribosomal protein P2 (RPLP2) and vesicle membrane protein (VAT-1) which are involved in ribosome and mitochondrial regulation respectively [33, 34]. This finding might indicate that SEA and ω-1 could affect cellular metabolism in DCs. Additionally, DC stimulated with SEA or ω-1 downregulated HLA-B and CD44 expression, which are important for efficient antigen presentation by DCs to T cells. The latter finding is supporting the dogma that Th2 differentiation may be favoured by a weak interaction between T cells and DCs at the immunological synapse [35, 36]. While this chapter pinpointed a set of proteins that may contribute to Th2 polarization by helminth-stimulated DCs, there are also indications that other factors, such as lipids, derived from helminths or induced by DCs upon recognition of helminths antigens, might be important for Th2 polarization by DCs.

All parasites contain lipids [37] which are not only of great importance to parasites as the chief form of stored energy, but also for their role as building units for cell membranes and various intracellular and paracrine signaling functions [38]. Parasite-derived lipids are not only important

for the biology of parasites themselves but might also affect immune polarization of the host. With this in mind, we performed a lipidomics analysis of the different life cycle stages of *S. mansoni* (**Chapter 4**). Although the lipid contents of different life cycle stages of *S. mansoni* has been studied [39], the lipid content of ES as well as the crude antigens has not been characterized. Using three complementary MS-based analytical platforms, our study has resulted in the establishment of a lipid profile database of both the different life cycle stages as well as what is found in extracts from *S. mansoni* that are often used for cellular immunological studies. This detailed lipid database can be used as a valuable resource to further study helminth biology in general and specifically that of *S. mansoni*. We found that different life cycle stages have distinct lipid signatures; for example diacylglycerols (DGs) are present predominantly in cercariae. DGs are important for promoting protein kinase C (PKC) activity, which regulates cellular function such as differentiation, cell growth and metabolism [40], processes that are known to be crucial for development of cercariae to worms [39]. In addition, a striking finding is the presence of prostaglandins (PGs) specifically in preparations from eggs (ES form eggs and soluble egg antigens). This ability to synthesize PGE_2 may not be limited to S. mansoni as it was recently found that also the whipworm *Trichuris suis* was able to secrete substantial amounts of PGE_2 [47]. Since egg deposition in the host during *Schistosoma* infection is strongly associated with Th2 induction [41] and members of PGs such as PGE_2 and PGD_2 have been shown to condition DCs for Th2 induction [42-44], this observation prompted us in chapter 6 to assess the role of these PGs in promotion of Th2 polarization by *S. mansoni* eggs.

In **Chapter 6**, we reported that that SEA not only contains PGE_2 but also stimulates DCs to synthesize PGE_2 and its isomers, and this occurred independently from ω-1. We additionally found that SEA binds to dectin-1 and dectin-2 on human moDCs, to trigger a signalling cascade involving Syk-Erk-$cPLA_2$ and COX1/2 to promote PGE_2 synthesis. Subsequently, our data shows, that this PGE_2 then acts in an autocrine fashion to drive OX40L expression on DCs to licence these cells to prime a Th2 response. In addition, we discovered that the non-enzymatic generation of PGE_2 isomers through reactive oxygen species (ROS)-driven auto-oxidation by DCs in response to SEA contributes to conditioning of DCs for Th2 priming. Finally, these findings were extended *in vivo*, showing that Syk and dectin-2 are essential for Th2 polarization *in vivo* as well as for Th2-driven granuloma formation around eggs in the liver during *S. mansoni* infection. Altogether, the data presented in this chapter reveal a previously unrecognized pathway that operates independently from ω-1 through which Th2 responses are induced by *S. mansoni*. Importantly, this pathway appears to play an instrumental role in the immunopathological outcome of schistosomiasis. The observations that antigens from *Fasciola hepatica* as well as from house dust mite have been shown to be recognized by dectin-1 [45] and dectin-2 [46], respectively, make it tantalizing to speculate that this dectin-PGE_2 dependent signalling axis in DCs is possibly not only triggered by *S. mansoni* but also by other helminths or allergens to promote Th2 responses.

To add to the complexity, increasing evidence implicates a role for microbiota in mediating immune regulation by helminths. A characteristic feature of helminth infections is that they elicit a type 2 immune response, alongside a regulatory response, especially in the setting of chronic, asymptomatic infections [48]. The stimulation of Treg activity has emerged as a central explanation certain microbiota and helminth infections, in ameliorating inflammatory diseases such as allergy and autoimmune diseases [49]. Interestingly, a recent publication showed a dependency of a gastrointestinal helminths on gut microbiota in modulating allergic inflammation [50], an effect that was mediated through the production of SCFAs by the microbiota. Although, microbiota are

the most well studied source of SCFAs, helminths are also known to generate acetate [51], which through specific enzymatic processes, can also be converted into butyrate, generally considered the most potent immunomodulatory SCFA [52]. Therefore, we aimed to elucidate the mechanism of action through which SCFAs induce regulatory responses in **Chapter 7**, by specifically focusing on the still poorly studied modulatory effects of SCFAs on human DCs. In this chapter, we demonstrate that butyrate suppresses LPS-induced maturation on DCs and conditions these cells to drive the differentiation of IL-10-producing type 1 regulatory T cells (Tr1). Mechanistically we found, butyrate to induce expression of RALDH by DCs, a key enzyme involved in retinoic acid (RA) production. This was required for both the priming of Tr1 cells and the maintenance of a tolerogenic DC (tolDC) phenotype. Finally, we observed that the conditioning of DCs by butyrate in our model depends on the combination of HDAC inhibition and GPR109A signalling. In chapter 7 for the first time we delineate the molecular mechanisms through which the SCFA butyrate conditions human DCs to prime Tregs. These findings do not only reveal additional mechanisms through which helminth may promote Treg activity in their hosts, but may also help in developing approaches to modulate regulatory immune responses for therapeutic purposes by targeting this butyrate-driven signalling pathway in DCs.
Key points of current knowledge and the advances described in this thesis regarding the molecular interplay between the immune system and helminths are summarized in Box.1, in section D and E respectively.

Future perspectives:

Uncovering novel mechanisms through which helminths modulate DCs

In the chapter 4 we identified different lipids in different cycle stages of *S. mansoni*. Some lipids are known for their capacity to induce Th2 such us PGD_2 and PGE_2 [42, 44]. However others lipids have not been investigated and the possible contribution of these other lipids to Th2 polarization awaits further investigation. Furthermore, how the helminths and microbiota can influence each other is still not fully understood. It has been postulated that antimicrobial peptides produced by helminths may shift microbial composition in the gut [51]. Moreover, it will be important to dissect the relative contributions of helminth- and microbiota-elicited Tregs in mediating immune modulation.

Entering unexplored territories in studying Th2- and Treg-priming by DCs in the context of helminth infections

Our proteomics (chapter 5) and lipidomics (chapter 6) studies have advanced our understanding of how DCs prime Th2 responses. For instance, in our DC-proteomics study, we have found the upregulation of protein HSP90AA1 in SEA- and ω-1- stimulated DCs, which is a protein important for type I interferon (IFN) production [53]. Interestingly, a recently published study showed that parasite-induced Th2 response depends on type I IFN signalling in murine skin DC [54]. This clearly warrants further investigation to assess whether this protein and type I IFN signalling in general is involved in Th2 priming by human DCs in response to *S. mansoni* antigens.

Evidence is accumulating that the capacity of immune cells to perform particular functions is controlled by their cellular metabolic state [55]. Also, the role of cellular metabolism in shaping the biology of immune cells that are involved in type 2 immune response is an active area of investigation. For instance, *in vitro* studies of Th2 metabolism showed that Th2 cell differentiation depend on glycolysis, and that inhibition of glycolysis interferes with the expression of GATA3 as well as IL-4 receptor [56, 57]. In contrast, to this date little is known about role of DC metabolism in

Th2 or Treg polarization, but would be a very interesting angle to uncover potential novel molecular mechanisms through which DCs prime these types of T cell responses.

From a methodological standpoint, we could foresee that the use of omics approaches will be crucial to fully unravel the molecular mechanisms through which DCs prime Th2 and Treg responses, as they allow for unbiased characterization of cellular phenotypes. In particular the integration of data from multiple omics approaches holds great promise to significantly move this field forward. For example, the integration of transcriptomics and metabolomics has resulted in the identification of distinct key metabolic pathways involved in classic and alternative activation of macrophages [58], which would not have been possible from analysis of either single omics approach.

Finally, a very important aspect to consider is that neighbouring cells may also influence the capacity of DC to induce Th2 polarization. As an example, B cells have been suggested to be important in the initiation of Th2 response by enabling the right localization of DCs in the lymph node [59]. Moreover, recent observations suggest that ILC2s contribute to Th2 responses by promoting DC migration, by interacting with T cells via MHC-II and by production of type 2 cytokines. This is an important concept as it suggests that studying Th2 polarization by merely looking at DC-T cell interactions, may be too simplistic as it fails to model the *in vivo* interdependency of multiple cell types to efficiently initiate Th2 responses [60, 61].

A summary of the direction for future research regarding molecular interplay of immune system and helminth infections are illustrated in figure 2 and summarized in Box.1 (section F).

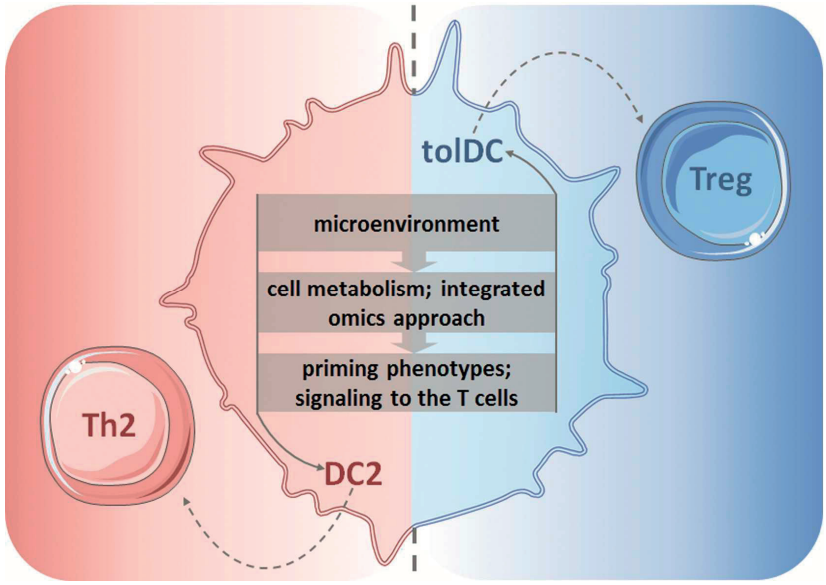

Figure 2. The proposed concept of how dendritic cells can be programmed to prime Th2 and Treg responses by helminths

DC biology is shaped by the microenvironment, including the cytokine milieu, signals from accessory cells, antigens and metabolites derived from helminth and microbiota. This will lead to changes in DC phenotype that can be studied by various integrated –omics approaches. Together this will determine the T cell polarization.

Box.1. Summary of the: current knowledge, contribution of the research in this thesis and future direction in the context of tracking helminths

	Molecular diagnosis	Immunological interplay
	A	**D**
Current knowledge	• Most of epidemiological studies of helminth infections have relied on microscopic techniques. • Study to study comparison based on microscopic diagnosis for helminth infections is challenging due to lack of standardization. • Real-time PCR-based diagnosis for detection of helminth infections has been shown to be more sensitive and specific than microscopy. • The current sample preparation following real-time PCR detection for gastrointestinal parasites is inadequate for detection of *T. trichiura*, which is one of most prevalent STHs.	▪ Helminth antigens can bind to receptors on DCs (including TLRs and CLRs) to modulate DCs for Th2 polarization via signalling-dependent and -independent mechanisms. ▪ ω-1 is a major antigen expressed by *S. mansoni* eggs that condition DCs for Th2 priming. However, it is not the only Th2-inducing component. Mechanistically, ω-1 is bound and internalized via its glycans by the MR and then interferes with the mRNA activity. ▪ Helminths can modulate host immune responses through promotion of SCFA production by microbiota ▪ SCFAs are metabolites produced by microbiota with known immunomodulatory potential.
	B	**E**
Contribution of this thesis	• Diagnosis based on DNA detection in stool seems a more reliable approach than stool microscopy to study the distribution of helminth infections and to compare different target populations (Chapter 2). • The bead-beating procedure, in particular in combination with ethanol preservation, prior to DNA extraction increases *T. trichiura* DNA yield in human fecal samples (Chapter 3).	▪ SEA and ω-1 alter the DC proteome, with the most pronounced effects on RPLP2 and VAT-1; as well as on proteins involved in antigen presentation (Chapter 5). ▪ Different *S. mansoni* antigens contain different lipid profiles, in which some lipids are known to be associated with Th2 induction(Chapter 4). ▪ *S. mansoni* egg antigens bind to dectin-1/2 on DCs to trigger a signalling axis involving Syk-Erk-cPLA$_2$ and COX1/2 that leads to PGE$_2$ and PGE$_2$ isomers synthesis, which in autocrine manner drive the expression of OX40L by DCs licensing these cell for priming of Th2 responses. (Chapter 6). ▪ This Dectin-Syk signalling cascade is crucially important for Th2 polarization and egg-driven granuloma formation by S. mansoni *in vivo* (Chapter 6). ▪ The SCFA butyrate conditions human DCs to prime IL-10 production Tr1 cells (Chapter 7). ▪ Butyrate depends on the combined action of HDAC inhibition and signalling via GPR109A to promote RALDH activity in DCs to license them to prime Tr1 cells (Chapter 7).
	C	**F**
Future research direction	• How can the operating procedures for helminth diagnosis, which include sample collection, handling, DNA extraction and detection, be standardized across the world? • How to develop a molecular-based rapid diagnostic tool to detect helminth infections?	▪ What is the role of and how does the microenvironment influence DC-mediated T cell priming? ▪ What are the mechanisms of interaction between helminths and microbiota and does their cross-talk lead to induction of regulatory immune responses? ▪ How can integrated –omics approaches be used to study of DC2 and tolDC biology? ▪ Do and how, helminth antigens affect the DC metabolism and does that contribute to their capacity in polarizing T cells?

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Addendum