Tracking helminths : from molecular diagnostics to mechanisms behind immune polarization

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Chapter 1

General Introduction

1. Helminth infections

Helminths are parasitic worms that live in their host for protection and to extract nutrients from, that allow them develop, grow, and reproduce. Among the 17 major neglected tropical diseases (NTDs), infections caused by soil transmitted helminths (STHs) and schistosomes are the most ubiquitous. In particular in tropical and subtropical areas, globally over 1.5 billion and 200 million people are infected with STHs and schistosomes, respectively [1, 2]. STHs are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), and two species of hookworms: *Necator americanus* and *Ancylostoma duodenale* [3]. The schistosome worms are *Schistosoma mansoni*, *Schistosoma heamatobium* and *Schistosoma japonicum* with *S. mansoni* the most prevalent [1].

Although people with light infections of STHs or *Shistosoma* spp normally have no symptoms, heavy infections are commonly associated with morbidity [4]. Using the disability-adjusted life years (DALYs) as a measure for burden of disease, STHs and schistosomiasis cause 5.2 million and 3.3 million DALYs, respectively, making them two major contributors to infection-induced DALYs [5]. Given that helminth infections can tremendously affect the quality of life, WHO together with several prominent global health organizations have implemented global deworming programs with the ultimate goal to eliminate these infections by 2020 [2].

Interestingly however, helminth infections do not only do harm. They are also known for their ability to dampen host immune responses, to allow their long term survival, and at the same time this can be beneficial to the host by helping to control excessive inflammation and thereby occurrence of inflammatory diseases. Several animal models and clinical trials have demonstrated beneficial effects of helminth infections on a broad range of diseases including celiac disease, inflammatory bowel disease (IBD), multiple sclerosis (MS), diabetes, asthma and atopy [6-11]. Therefore, besides combating helminth infections worldwide, investigating how helminths and molecules derived from these parasites can modulate the immune responses will be equally important.

2. Detecting helminth infections: moving toward a standardized molecular based diagnosis technique

Diagnosis of intestinal helminths has always relied on microscopy-based detection of egg, larva or cysts in human faecal samples. Although microscopic methods are sufficient to detect the most prominent parasite species with a high prevalence and load of infection, it is important to realize that there is currently no single method available that can diagnose all helminth infections with high sensitivity and specificity. Moreover, the current shifts in parasite distribution and successful results from control programmes increase the need for more sensitive and high-throughput diagnostic procedures to identify the remaining infectious reservoir [12]. For monitoring the progress of control or elimination programs and ongoing surveillance of helminth infections, new standardized diagnostics are critically important [2].

The use of real-time PCR for diagnosis of intestinal parasites has been implemented in several endemic countries as well as in clinical laboratories [13]. Real-time PCR has demonstrated to outperform microscopy in the detection of all helminths under study thus far [14-20]. However, further validation is necessary, such as direct comparison of the outcome between different endemic countries using the same pipeline (i.e. sample collection, DNA extraction methods and real-time PCR detection). Nonetheless, it is clear that real-time PCR holds promise as an alternative diagnostic tool and that it could ultimately become the first-line standardized screening method for the diagnosis of helminth infections.

Despite the rapid development and great potential of molecular based techniques to diagnose helminth infections, there are still some hurdles that need to be overcome before it could be used as standardized diagnostics both in the field and in the clinic. First, the need for technologically equipped laboratory to perform real-time PCR [21] makes this method currently mainly possible in research settings [22]. Moreover, current sample preparation techniques for DNA isolation are insufficiently optimized to detect all intestinal parasites. For instance, additional bead-beating treatment prior to DNA extraction has been suggested to improve *T. trichiura* DNA extraction [19]. However, population based data from high prevalence area is still lacking, making it difficult to evaluate the performance of the bead-beating. It is worth mentioning that the effect of bead-beating treatment on other intestinal parasites needs to be evaluated as well to assure that bead-beating will not affect the detection of other intestinal parasites.

3 Helminth-host immune system interplay

The immune system protects organisms from infections with layered defences of increasing specificity. The innate immune system provides an immediate but non-specific response and the long-term protection relies on adaptive immune responses mediated by T cells and B cells. Parasitic helminths have evolved together with the mammalian immune system and as a consequence they have developed numerous survival strategies that include secretion of molecules that can modulate the host immune system [23]. A key component of the immune system that has evolved to minimize the virulence of helminths is the type 2 (or T helper 2) immune response [24].

The generation of type 2 immune responses involves innate and adaptive immune cells as well as non-hematopoietic cells such as epithelial cells as illustrates in Figure 1. Upon infection with helminths, the first cells to be exposed to these pathogens are in many cases epithelial cells that start to produce a variety of type 2 alarmins and cytokines which include thymic stromal lymphopoietin (TSLP), IL-33, resistin-like molecule (RELM)-β and IL-25, of which the latter two are predominantly produced in the gut tuff and goblet cells. In response to TSLP and IL-33 mast cells and basophil are recruited from circulation to undergo degranulation releasing protease, histamine, IL-4 and TNF which can amplify type 2 inflammation and recruitment of leukocytes. Moreover, IL-33 and IL-25 are important for actviation of type 2 innate lymphoid cells (ILC2). Activated ILC2 secrete IL-5 and IL-13, which in turn induce eosinophilia and mucus secretion, respectively. Immature dendritic cells (DCs) reside in the peripheral tissues where they take up helminths antigens, undergo maturation while migrating to lymph nodes and then skew naive T cells toward a T helper 2 (Th2) profile which is characterized by production of IL-4, IL-5, IL-9 and IL-13. Additionally, alarmins such as TSLP also signal to DCs to express Th2-recruiting chemokines and to condition them to prime Th2 responses. Exposure to IL-4 and IL-13 skews macrophages towards a tissue repair phenotype, termed M2 or alternatively activated, characterized by the expression of factors such as arginase (Arg) 1, shitinase-like protein YM1 and RELM-α. Finally IL-4 produced by T cells is involved in B cell activation and Ig class switching towards IgE and IgG1 (in mice) or IgG4 (in humans).

Dendritic cells (DCs) are key players in bridging innate and adaptive immunity. Just like their crucial role in inducing Th1 and Th17 responses against intracellular pathogens (virus and bacteria) and extracellular unicellular pathogens (fungi and bacteria), respectively, the role of DCs in Th2 induction against helminth parasites is indispensable. In mouse models in which

CD11c^{+}DCs were depleted, Th2 priming in response to helminth infections was severely impaired [25-27].

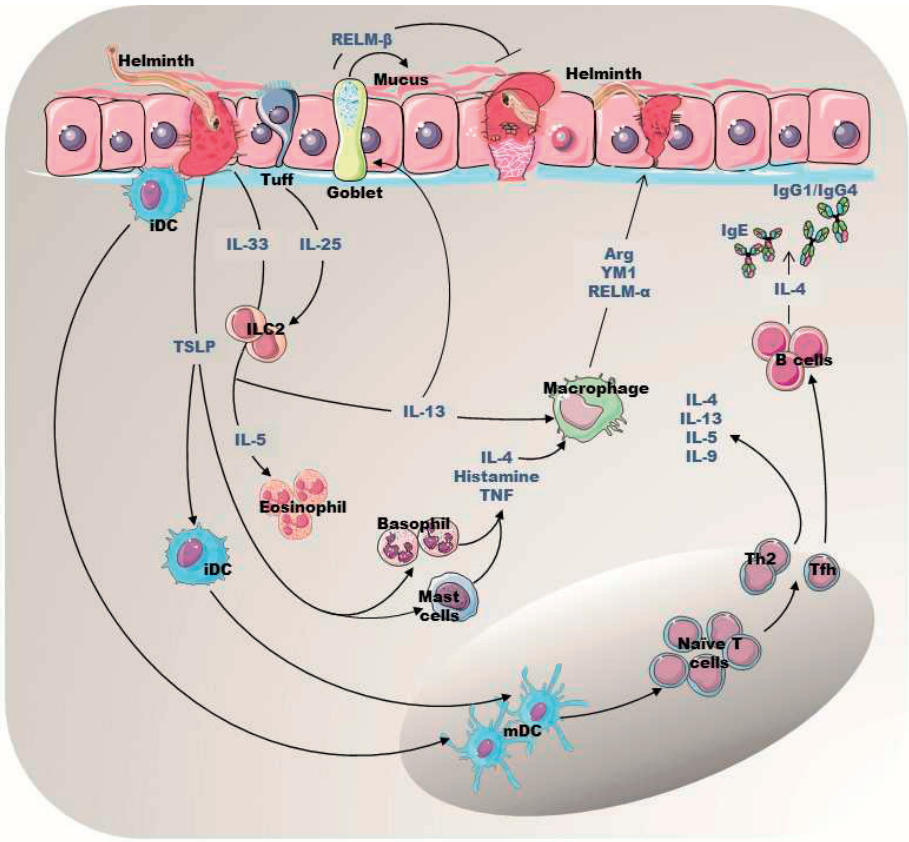

Figure 1. Type 2 immune responses induced by helminths
Helminth infections leads to the tissue damage and secretion of the cytokines IL-33 and TSLP. IL-33 signals on ILC2 leading to recruitment of eosinophils through the secretion of IL-5. ILC2 also sense the increased production of IL-25 by tuff cells and secrete IL-13, which feedback on goblet cells leading to increased mucus production. In response to TSLP, mast cells and basophils are recruited and start amplifying the type 2 inflammation by producing IL-4, histamine and TNF. Subsequently IL-13 and IL-4 contributed to skew macrophage in to M2 phenotype and produce RELM-α, Arg and YM1, which are important for tissue repair. Meanwhile, immature DC resides in the peripheral tissues take up helminths antigens or by sense the TSLP, undergo maturation while migrating to the lymphoid organs to polarize naive T cell into Th2. Th2 cell is characterized by the production of IL-4, IL-5, IL-9 and IL-13. Lastly, T follicular helper cells provide help to B cells for IgE and IgG production in IL-4 dependent manner.

Despite the well appreciated role of DCs in initiation of Th2 responses, several questions regarding the mechanisms underlying this response await to be fully addressed: first, what are the molecule(s) from helminths that is(are) responsible for Th2 priming and how are they recognized by DCs. Second how do these molecules condition DCs to induce Th2 responses. And finally, what are the characteristics of helminth-conditioned DCs that enable them to drive Th2. The current knowledge of these three aspects, will be summarized in the following sections and is schematically shown in Figure 2.

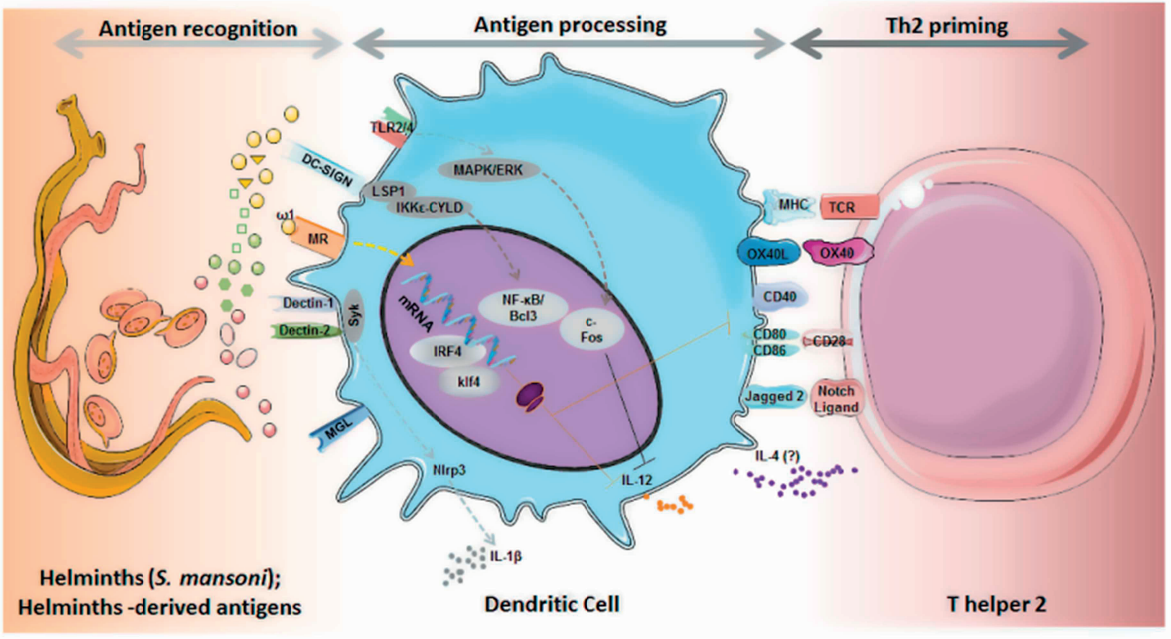

Figure 2. Molecular interaction involved in DC conditioning Th2 polarization by helminth

Antigen recognition: Helminth and helminth derived-antigens are recognized by DC via TLRs (TLR2 an TLR4) and CLRs (MGL, DC-SIGN, MR, Dectin-1 and -2). Antigen processing: Depending on helminth-derived antigens encountered via specific ligation of PRR results in; phosphorylation of MAPK/ERK which stabilized c-Fos and result in suppression of IL-12 production; phosphorylation of Syk that activate Nlrp3 which mediates IL-1β secretion; phosphorylation of LSP1 and subsequently the recruitment of IKKε-CYLD complex activate Bcl3/NF-κB which is important for controlling the CD40 and Jagged 2 expression; Klf4 is required for controlling the transcription factors necessary for Th2 induction in $IRF4^{+}$ DC; in parallel, ω-1 internalized by MR degrades rRNA and mRNA in RNase dependent manner, leading to inhibition of protein synthesis including co-stimulatory molecules (CD80 and CD86) as well as IL-12. Th2 priming: Altogether, via multiple signals which are: PPR-mediated interference of antigen presentation on MHC-II; lower expression of classical maturation markers; promotion of OX40L as well as Jagged 2; lower IL-12 production; and at last, possibly the production of IL-4 by DCs which is still questionable, thus all mediate Th2 polarization.

3.1. Recognition helminth-derived molecules by dendritic cells

Soluble *S. mansoni* egg antigen (SEA) and crude extracts prepared from nematode and cestode parasites [23] are often used in *in vivo* and *in vitro* models to study Th2 polarization by DCs. In addition, several excretory/secretory (ES) fractions from helminths have been found to harbour Th2 polarizing molecules [23, 28] and importantly, within those fractions a couple of helminths-derived single compounds with Th2-inducing capacity have been characterized. For instance a worm-derived lipid secreted by a roundworm, *Acanthocheilonema viteae*, termed ES-62 can condition DCs to prime a Th2 response [29]. Furthermore, lyso-phosphatidylserine (PS) derived from schistosome and *Ascaris* worms was shown to promote Th2 via DCs [30]. Finally an important contribution to this field was made by the discovery of omega-1 (ω-1), a single glycoprotein secreted by *S. mansoni* eggs which was found to harbour strong Th2-polarizing capacity [31, 32].

DCs are equipped with so-called pattern recognition receptors (PRRs) that are important in recognizing pathogens-associated molecular patterns (PAMPs). These receptors encompass several families including Toll-like (TLR), C-type lectin (CLR), nucleotide-binding oligomerization domain/NOD-like (NLR) and RIG-1-like (RLR) receptors [23]. Two out of 11 well-characterized TLRs in human, TLR2 and TLR4 are shown to be essential for Th2-specific DC response to SEA or PS from *S. mansoni* and ES-62 or phosphorylcholine (PC) from ES-62, respectively [29, 33, 34]. However, there is evidence suggesting that TLRs signalling might be dispensable for Th2 induction by helminth as shown by bone marrow-derived DCs (BMDCs) from TLR-2 and TLR-4 KO mice that can still induce a Th2 response [35]. CLRs, including DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), macrophage galactose binding lectin receptor (MGL) and mannose receptor (MR), have been shown to be involved in the internalization of SEA [36]. Another DC CLR that has been shown to bind SEA is dectin-2. Although dectin-2 is primarily known for its ability to recognize fungal antigens, the complex of dectin-2 and FcRγ on BMDC was required for SEA-induced production of IL-1β [37]. Studies have shown that CLRs expressed on DCs that sense helminths glycans, play important role in Th2 induction. For instance, glycan found in SEA Lewis^X (Le^X) which contains Lacto-N-fucopentaose III (LNFIII) has been shown to bind to TLR4 as well as DC-SIGN [36]. DC-SIGN was also shown to be able to recognized glycans from *Toxocara canis* [38]. Another example is, ES from *Taenia crassiceps* (TcES), which can bind to MR and MGL on BMDCs and induce Th2 polarization in a glycan-dependent manner [39]. More recently, a study demonstrated that MR on DC is responsible for the binding and internalization of the *S. mansoni* secreted glycoprotein ω-1 [40]. Thus so far, the evidence suggests helminths glycans sensed by CLRs are generally important molecular patterns for Th2 induction by DCs.

3.2. Signalling by helminth-derived molecules in dendritic cells

Classically, TLR signalling involves the activation of mitogen activated protein kinases (MAPK) including p42/p44 (extracellular signal-regulated kinase, ERK1/2), Janus Kinase (JNK) and p38. Strong release of IL-12 by DC that favours Th1 differentiation is the result of p38 phosphorylation. In contrast, SEA and PS favour phosphorylation of ERK which is associated with induction of Th2, via stabilization of the c-fos transcription factor that suppresses IL-12 release [30, 41]. Likewise, ES-62 and LNFPIII were also shown to induce the activation of ERK in DCs via TLR4 ligation [42-44]. Moreover, a role for ERK in Th2 response development is indicated by the findings that ERK$^{-/-}$ mice exhibit increased susceptibility to experimental autoimmune encephalomyelitis and are Th1 prone. Together these suggest that ERK may play an important role in Th2 polarization [45].

However, the PRR that drive ERK activation in response to helminths and helminths-derived antigens including SEA stimulation remain to be elucidated.

Signalling via NF-κB was also shown to be involved in Th2 response. For instance, mice with the BMDCs from which NF- κB knockout failed to instruct Th2 cell differentiation in response to SEA and LNFPIII [46, 47]. Recently it was shown that Le^X residues derived from SEA can be recognized by DC-SIGN on moDCs. Thus recognition promotes the phosphorylation of LSP1 subsequently the recruitment of IKKε-CYLD that lead to the activation of Bcl3, atypical NF-κB family member which is crucial for inducing a Th2-priming phenotype [48]. Finally, SEA can activate the Nlrp3 inflammasome and increase IL-1β release by BMDCs via spleen tyrosine kinase (Syk) downstream of dectin-2 [37]. However, to what extent this pathway in DCs regulates Th2 priming remains to be investigated. With regards to the signalling events induced by single molecules from helminths, ω-1 was found to modulate DC function for induction of Th2 responses. Mechanistically, ω-1 interferes ribosomal function and protein synthesis in an RNase-dependent manner, following MR-driven translocation into the cytosol [40].

3.3. T helper 2 priming by dendritic cells

Proper priming and polarization of Th responses by DCs is dependent on three DC-derived signals: 1, presentation of cognate peptide antigens in the context of major histocompatibility complex (MHC) II; 2, co-stimulation; and 3, expression of T cell-polarizing factors. The nature of this latter signal is generally decisive for Th differentiation fate. A classic example is IL-12 secretion by DCs that promotes Th1 polarization. However, such a polarizing signal for priming of Th2 responses by helminths has remained elusive. This initially led to the so-called default concept which states that the absence of a Th1-priming signal will promote Th2 polarization. However, IL-12 deficient mice exposed to microbial pathogens failed to default to a Th2 pattern [49], indicating there are active signals involved in this process. A recent transcriptomics study has shown type I interferon to be a key transcriptional signature of murine skin DCs that enables them to drive Th2 induction in response to helminth antigens [50]. However, whether there are groups of type I interferon specifically associated with Th2 polarization or rather as the common signatures similar to Th1, thus need further studies. Another question is, whether the role of type I interferon is tissue and/or cell specific in terms of mounting a Th2 response by helminths. IL-4 can skew naive T cells toward Th2, but despite the importance of this signal, its origin is an area of much debate because it is still debated whether DCs can produce IL-4 [51-53]. It is possible that IL-4 or other Type 2 cytokines that can promote Th2 differentiation, originate from non-DC sources like ILC2s [25], that in conjunction with signal 1 and 2 from DCs can provide the necessary cues for Th2 differentiation.

Alterations in signal 1 and/or 2 have also been linked to Th2 polarization in the context of helminth infections. For instance, studies of early signalling events have shown that short-term or low-avidity interaction between DC and T cell favour Th2 differentiation due to low (avidity) antigen presentation (signal 1) [54]. Interestingly, this phenomenon has also been described for DCs exposed to SEA or ω-1 [31, 55]. Expression of CD40, as part of signal 2, is known to be important for Th2 induction by *S. manoni* [56]. Moreover, SEA has been shown to alter the balance between expression of co-stimulatory molecules CD80 and CD86 on DCs, which has been linked to favouring Th2 polarization as well [57]. Although DC expressing the notch ligand jagged2 have been implicated in Th2 priming, the expression of this molecule is not essential for Th2 response upon SEA stimulation, as Jagged-2-deficient BMDCs are still able to induce Th2 in response to SEA [58]. Finally, several groups have shown that in response both to SEA or TLSP, DC upregulated the

co-stimulatory molecule OX40 ligand (OX40L). T cells sense OX40L on DC through OX40 receptor and produce IL-4, IL-5 and IL-13 [59-63]. Although it has been shown that SEA-treated OX40L-KO DCs are still able to induce a Th2 responses, Th2 cell expansion was reduced, suggesting that the OX40L-OX40 signal contributes to the Th2 response by allowing for optimal expansion of Th2 cells [60].

The mechanisms behind the ability of helminth antigens to modulate DCs for priming of Th2 responses have been primarily studied in *in vitro* DC models, which fail to capture the complexity of the different DC subsets that exist *in vivo*. Recent studies have demonstrated the existence of a specific Th2-priming DC subset that is developmentally dependent on transcription factor (TF) IRF4 and Klf4 and is required for priming of Th2 responses including those in response to helminth antigens [64-66]. Future studies will be needed to determine the relative contribution of the ability of helminth antigens to conditions DCs for Th2 priming versus the intrinsic ability of certain DC subsets to prime Th2 responses, in *in vivo* upon helminth infections.

4. Interaction with microbe metabolites: the extended niche

4.1. The relationship between helminths and microbe

In recent years it has become increasingly clear that our microbiota play a key role in shaping our immune system. Given the fact that many helminths and microbiota inhabit the same niche, the intestine, it is likely that they interact and impact on each other [67]. Beside the ability of helminths to secrete a variety of products that directly affect host immune function, they also have the capacity to influence the composition of the microbiota [68-71], thereby possibility regulating immune function indirectly. *Vice versa,* changes in microbiota can affect susceptibility to helminth infection [70, 72, 73], indicating that the crosstalk between these two groups of endobiota can play an essential role in host intestinal immune function and homeostasis [74]. Importantly, this relationship between microbiota and helminths has been shown to have beneficial effects on inflammatory diseases including IBD, celiac diseases, multiple sclerosis, diabetes and allergy [67]. As an example, a recent study showed that the intestinal microbiota contributes to the ability of helminths to suppress allergic inflammation [75]. With respect to host defenses, helminth infections elicit type 2 immune responses as described in the previous section, while opportunistic bacterial infections can evoke Th1 or Th17 responses [74, 76, 77]. However, both microbiota and helminths have the additional capacity to promote regulatory immune responses, which are key to limit inflammation and associated tissue damage that could be caused by the aforementioned effector responses when left unchecked. The potential interaction between microbiota and helminths is illustrated in Figure 3.

4.2. Immune modulation by the bacterial metabolites

The human body is colonized by roughly 3.9×10^{13} bacteria [78] that can potentially interact with the immune system. There are more than 50 bacterial phyla, but human gut-associated microbiota is dominated by four main phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* [79]. Microbiota are able to produce numerous metabolites, among them are the short chain fatty acids [80]. A significant number of studies have highlighted the immunomodulatory potential of SCFAs with beneficial effects on a broad range of inflammatory diseases IBD, colitis, asthma, obesity and arthritis [80-84]. Mechanistically, SCFAs can affect immune cells via signaling through specific G protein-coupled receptors (GPRs) [85, 86] as well as through histone deacetylation (HDAC)

inhibition. This primarily leads to suppression of inflammatory responses (i.e. reduction in expression of pro-inflammatory cytokines) by DCs, macrophages and T cells [87-89]. With regard to DCs, it has been shown that SCFAs can promote tolerogenic properties in DCs resulting in increased ability to prime Treg cells [80, 86]. However, the exact molecular mechanisms through which SCFAs modulate the capacity of human DCs to induce Treg cells is not known.

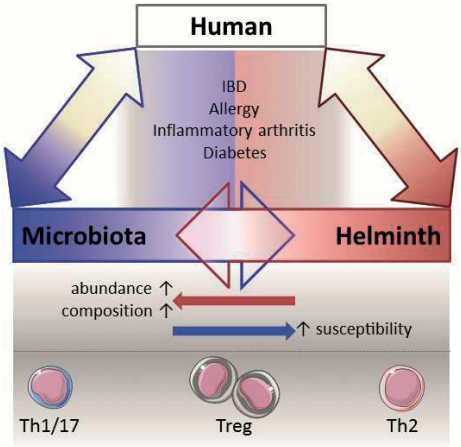

Figure 3. Interactions between human host, helminths and microbiota
Microbiota and helminths are co-inhabit the human gut with multi-way interactions. Helminths infections appear to be correlated with heightened microbiota diversity, and conversely, alteration of microbiota composition and abundance can alter susceptibility to helminths. Mechanistically, microbiota induce the Th1/Th17, whereas helminths are strong Th2 inducers. Moreover, both are capable of inducing Treg to keep immune responses under control. Thus can lead to protection against inflammatory diseases, such as IBD, diabetes, allergy and inflammatory arthritis.

5. Scope of the thesis

This thesis targets helminth infections and how they could affect the immune system in order to devise interventions that can help, on the one hand to control helminth infections and on the other to modulate inflammatory diseases. To start with, research is still needed to detect helminths with utmost sensitivity. Hence, the first two chapters of this thesis are focused on molecular diagnostics of helminth infections. Specifically, in **Chapter 2** the performance of real-time PCR *versus* microscopy in diagnosing STH infections are compared. The studies are performed in two STH endemic areas namely Beira, Mozambique and Nangapanda, East Nusa Tenggara, Indonesia. In **Chapter 3** a study is described that aims to improve the diagnosis of *T. trichiura* by optimizing sample preparation prior to DNA extraction from faces. The optimized procedures are then compared to the standard procedure.

Next, the issue of immune modulation by helminth infections, still requires in depth understanding of how helminths induce Th2 and Treg responses before new intervention strategies can be developed to combat these infections and to manipulate inflammatory diseases. Therefore the second part of the thesis deals with the interplay between helminths and the mammalian host immune system with particular emphasis on the mechanisms by which helminth-derived molecules promote Th2 responses. First, in **Chapter 4**, the lipid profile of different life cycle stages of *S. mansoni* is described, that can serve as a starting point to identify potential parasite lipid mediators that influence the host immune response. In **Chapter 5** the proteome of moDCs stimulated with helminth antigens is characterized using a mass spectrometry-based method to link expression of certain proteins by helminth-conditioned DCs to their ability to promote Th2 differentiation. As follow up to **Chapter 4**, the role of lipids, specifically prostaglandins, in SEA-driven Th2 polarization by DCs is studied in **Chapter 6.** Finally evidence is

accumulating that helminths interact with bacterial communities in the host, thereby shaping the SCFA profile these bacteria produce, which may impact immune cell function including DCs. Therefore, **Chapter 7** characterized the molecular mechanisms through which SCFAs produced by microbes condition human DCs to prime regulatory T cells.

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