CHAPTER 1

General Introduction
Scope & aims of this thesis

Background

Helminth (a Greek word for 'worm') infections are a major – and neglected – public health problem. Chronic helminth infections are thought to induce a regulatory network in the host that prevents their elimination, on the one hand, while protecting the host against the pathological consequences of excessive inflammation, on the other[1]. Importantly, this downregulation is not only directed against helminth antigens, but can also extend to other antigens: so-called bystander suppression. This may have significant consequences for immunity to incoming infections, such as malaria[2], and for responses to vaccines administered to helminth-infected subjects[3]. At the same time, several studies have suggested a protective effect of chronic helminth infections on atopy and allergic diseases[4]. Moreover, there are indications that anti-inflammatory properties of helminths might be beneficial in dampening the excessive inflammation observed in chronic autoimmune and other inflammatory diseases[5]. To be able to translate these epidemiological findings into therapeutic strategies, it is essential to unravel the mechanisms underlying detrimental and beneficial effects of helminths. Since it is thought that immunological features of host parasite interaction during helminth infections could be responsible for these epidemiological findings, we set out to characterize the immune regulatory network associated with helminth infections.

Coevolution of humans and helminths

Currently, helminths affect millions of people worldwide, mostly inhabitants of rural areas in low- and middle-income countries[6]. The different species of helminths are classified into two major groups, nematodes and platyhelminths, or roundworms and flatworms. The nematodes include soil-transmitted helminths (STH), causing intestinal worm infections, and filarial worms, which lead to lymphatic (lymphatic filariasis, LF) or subcutaneous manifestations (onchocerciasis and loiasis). Platyhelminths are further divided into trematodes, such as schistosomes, and cestodes or tapeworms. Helminth-induced mortality is low, compared to other tropical diseases such as malaria, however the chronic presence of worms can have a major impact on health by affecting host nutrition, growth and cognitive development, which can be substantially impaired by chronic helminth infections[7]. In addition, although majority of infections with these parasites do not lead to noticeable immunopathologies, in a subset tissue pathology can cause significant disabilities, for example elephantiasis resulting from LF or liver granulomas formed around schistosome eggs.

Parasitic worm infections are likely to have been with us throughout evolution. In recent history, schistosome eggs were identified in Egyptian mummies that are approximately 3000 years old[8]. In many communities in endemic areas, people of all ages can be infected with helminths without much outward clinical signs of

infection. This has often been interpreted as a peaceful coexistence of worms with their human host. The coevolution of worms and humans has taught us not only that these parasites are endowed with immune evasion mechanisms, but also that helminths, more profoundly than other pathogens, have directly altered the host's genetic composition, as shown by research into the evolution of human interleukin (IL) genes[9]. Parasite richness in an environment has been shown to be correlated with various single nucleotide polymorphisms (SNP) in IL genes, indicating that helminths can act as a selective pressure to shape the immune system. These genetic alterations, while beneficial when humans are parasitized by helminths, might be detrimental when these parasites are eliminated.

Nowadays, parasitic infections have been largely eradicated in affluent countries due to improved sanitation, control of water bodies and housing. Although this can be regarded as a great accomplishment of the 20th century, the question has arisen as to whether the presence of helminth infections might have some beneficial aspects. The hygiene hypothesis was based on Strachan's publication on the negative association between hay fever incidence and the number of – especially older – siblings in a household, which was thought to reflect the burden of infection in childhood[10]. The hypothesis stated that the increase in atopic diseases in high-resource settings might be due to the improved hygiene and the reduction in childhood infections. The hygiene hypothesis has been extended to other diseases that stem from immune dysregulation such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, diabetes mellitus and cardiovascular diseases[11]. Whereas Strachan proposed that respiratory viral infections contribute to the observed protection against hay fever, it has become clear that other infectious agents, such as helminths, which are able to modulate the immune system and establish chronic infections in their human host, may also be associated with less allergies and other inflammatory diseases.

The immune regulatory network

It has been hypothesized that helminths are able to induce an immune regulatory network, which can suppress the host immune system in such a way that the parasite is not expelled and the host tissues are not damaged too extensively[12]. Unresponsiveness in lymphocyte proliferation to helminth antigens was already described in the 1970s for individuals with *Schistosoma mansoni* infection as well as for those with bancroftian filariasis[13,14]. Studies in lymphatic filariasis indicated that an adherent cell population within peripheral blood mononuclear cells (PBMC) could suppress anti-filarial immune responses[15]. Moreover, the T cell compartment was dominated by suppressor T cells, removal of which augmented lymphocyte proliferative responses[16].

At the same time, in the field of autoimmune diseases, it was recognized that diseased individuals were often affected by more than one autoimmune condition[17,18], leading to the idea that suppression of auto-reactive T cells could be mediated by a common mechanism. A landmark publication from Sakaguchi and colleagues showed that depletion of T cells expressing CD25, the α-chain of the IL-2 receptor, led to a range of autoimmune disorders in mice[19], whereupon several studies in humans indicated that $CD4^+CD25^+$ cells can also exert suppressive activities[20]. Another breakthrough reported by the same group was the discovery that transduction of T cells with the transcription factor Forkhead box protein 3 (Foxp3 for rodents, FOXP3 for humans) could prevent autoimmune gastritis and inflammatory bowel disease in a mouse model[21]. Mutations in the *Foxp3* gene had earlier been identified as the cause for scurfy mice and the human equivalent, immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), both characterized by multiple autoimmune and inflammatory processes[22,23]. TGF-β has been identified as the main cytokine capable of inducing Foxp3 expression in naïve T cells[24]. The subsequent generation of Foxp3-GFP reporter[25] and "depletion of regulatory T cell" (DEREG)[26] mice has enabled an exponential number of studies on the function of these regulatory T cells (Tregs) in autoimmune, allergic, infectious and malignant diseases as well as in transplantion tolerance.

Thus, cells coexpressing FOXP3 and high levels of CD25 were regarded as a novel T cell subset, termed Tregs. Soon thereafter these were called 'natural' Tregs, since these cells are thought to originate from the thymus and are naturally present in the periphery to maintain self-tolerance[27]. In addition, several other T cell subsets with regulatory activity have been described, which may or may not express CD25 or FOXP3. These subsets are jointly named adaptive or inducible Tregs, since these cells can be induced through specific antigenic stimulation[28]. For example, T cells in the periphery can be induced to express FOXP3 capable of suppression, T-regulatory-1 (Tr-1) cells secreting IL-10 and transforming growth factor (TGF)-β were demonstrated to have a regulatory role[29] as well as Th3 cells, which were originally identified as TGF-β secreting cells that could mediate oral tolerance to myelin peptides in MS patients[30]. Furthermore, some studies have described CD8-positive Tregs[31] and even $CD4^-CD8^-$ Tregs[32], however these subsets have not been characterized in much detail.

The mechanisms for Treg-mediated suppression are not fully understood in humans, but research in animals and *in vitro* models has made great progress in the last decade[33,34]. Since CD25 was the first surface molecule implicated in cells involved in self-tolerance[19], high consumption of IL-2 by Tregs ("cytokine sink") was suggested as a way of depriving other T cells from this critical factor and thereby leading to apoptosis[35]. A well-characterized cell contact-dependent mechanism of suppression by Tregs is through the expression of inhibitory

molecules. CTLA-4 is the suppressory equivalent of CD28 expressed on T cells and is involved in costimulatory interactions with CD86 and CD80[36]. Importantly, agonistic CTLA-4 antibodies are now one of the options that can be used for therapeutic intervention to inhibit excessive T cell activation in rheumatoid arthritis[37]. Next to cell-contact mediated suppression, Tregs can also exert their regulatory functions by secreting immune modulatory molecules. IL-10 and TGF-β are the best-known examples of suppressory cytokines. Another cytokine lately added to this spectrum is IL-35, which was shown to contribute to the human regulatory network and possibly infectious tolerance[38]. Moreover, the expression of tumor necrosis factor (TNF) receptor II (TNFRII) has been observed in rheumatoid arthritis and malaria infections, with possible mode of actions being enhancement of Treg activity in TNF-rich environments and neutralization of TNF[39,40]. Recent work has further identified CD39 and CD73 co-expression on Tregs as part of their suppressory activity[41]. These ectoenzymes generate adenosine, which inhibits T cell proliferation and activation via the A2 adenosine receptor (A2AR) pathway.

Characterization of Tregs during helminth infections

Experiments in murine models have paved the way for studies that investigate the contribution of Tregs in human helminth infections. Although murine models, which provide homogenous genetic and environmental background, are expected to provide robust studies of infections, the results obtained have not always been clear-cut. For example regarding the role of IL-10 in murine schistosomiasis (*S. mansoni*), $CD4^+CD25^+$ cells expressing Foxp3 were able to inhibit Th1 cell proliferation through IL-10[42]. In another study IL-10 from Tregs was shown to be more important for host survival than IL-10 produced by other cell subsets[43]. However, other studies using the same models have shown that IL-10 is not essential for Treg-modulated suppression of Th1 or Th2 responses[44,45]. These discrepancies illustrate the degree of the complexity of the so-called "regulatory network" and the possible relation to parasites.

The group of Rick Maizels has contributed much to unraveling the cellular mechanisms of the immune regulatory network in murine models of filariasis (*Brugia malayi* or *Litomosoides sigmodontis*) and intestinal helminth infections (*Heligmosomoides polygyrus*). Although these models are very different, in all three an expansion of $Foxp3^+$ Tregs during early stages of infection was seen[46-48]. In successive studies, it has been shown that Tregs during *L. sigmodontis* infection suppress anti-parasite immunity through surface expression of CD25 and glucocorticoid-induced TNF receptor family related gene (GITR)[48], that Tregs in *H. polygyrus*-infected animals are more suppressive *in vitro* than those from uninfected mice[46], that adult parasite stages also induce Foxp3 expressing T cells

which have *in vitro* suppressive capacity which extends to bystander antigens[47] and finally, that *in vivo* CD25 depletion 7 days prior to infection could prevent the appearance of microfilaremia and reduce worm burden, indicating that Tregs suppress parasite killing *in vivo*[49]. *In vivo* and *in vitro* anti-CD25 treatment has been used in several other studies to deplete Tregs, leading to increased anti-parasite responses in murine infections with *Brugia pahangi*[50] and *Schistosoma mansoni*[43-45], but this same treatment did not enhance immune responses in other studies of *S. mansoni*[51] and *Trichinella spiralis*[52]. Another approach is depletion of Foxp3$^+$ Tregs by using DEREG mice. Expression of diphtheria toxin (DT) receptor fused with GFP under control of the *Foxp3* locus enables complete depletion of Foxp3$^+$ Tregs by administration of DT[26]. Interestingly, it was noted that only depletion of Tregs in the first days of infection is effective in improving resistance to worms. In a *Strongyloides ratti* mouse model, this method was applied and early depletion of Tregs increased type 2 responses and reduced worm burden significantly but did not change intestinal pathology, whereas depletion at a later time point (4 days after infection) had no effect on worm burden or pathology[53]. In a model of *H. polygyrus* infection however, the same Treg depletion applied at 4 days post-infection, while enhancing Th2 responses, did not affect the numbers of worms and exacerbated intestinal pathology[54]. Taken together, these data suggest that anti-CD25 treatment and Foxp3$^+$ Treg depletion show similar results; Tregs are rapidly increased upon helminth infection and are in particular important in inhibiting protective immunity at early stages of infection, whereas their effect on intestinal pathology is not consistent (summarized by Taylor et al.[55]).

In human infections, the characterization of Tregs has been more complicated (Figure 1). Up until recently, the presence of Tregs was shown indirectly by mRNA analysis of total peripheral blood mononuclear cells (PBMC)[56,57], but flow cytometry has now been established as the principal method for Treg identification during helminthic infections[58,59], however staining for FOXP3 was not available until recently. Since FOXP3 is an intracellular protein, expression can only be analyzed in fixed cells with a certain staining protocol[60]. Therefore, if isolated Tregs are needed, only surface molecules, which are less specific for Tregs, can be used for their identification and sorting[61]. Furthermore, large volumes of blood are needed for the purpose of isolating Tregs from peripheral blood, which may not always be feasible. Finally, the most state of the art methods for Treg characterization demand well-equipped laboratories with specialized operators that are more than often limited in low-resource settings. In the field of helminth research, we are therefore restricted to indirect methods of assessing Treg properties, such as phenotypic analysis of markers and depletion of Tregs in *in vitro* cell cultures (Figure 1).

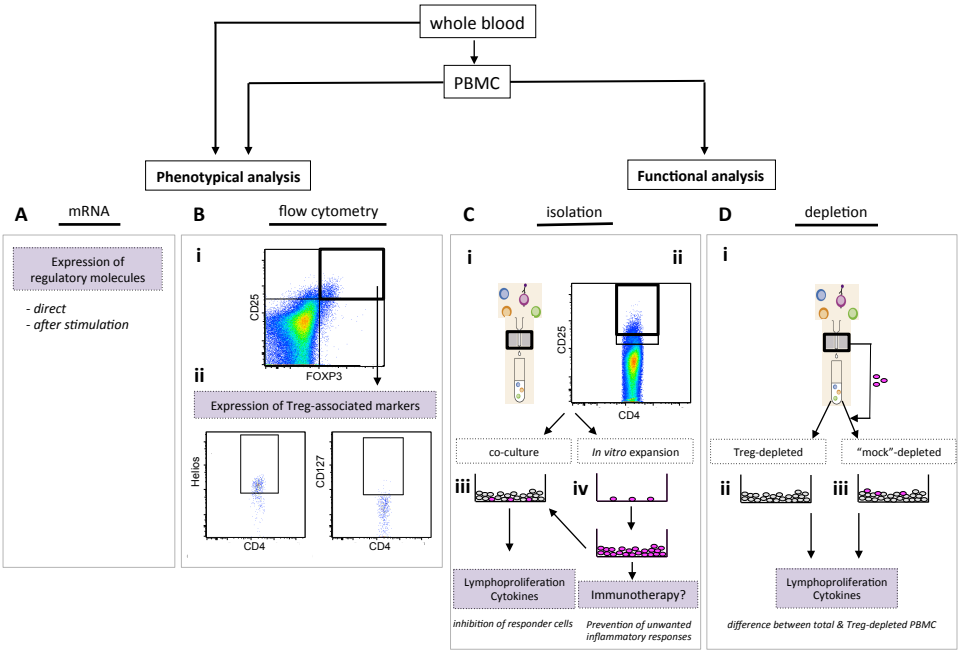

Figure 1. Different methods of characterizing human Tregs. Schematic representation of the different ways Tregs have been analyzed. In A and B, the methods of phenotypic characterization are summarized. (A) Whole blood as well as PBMC can be assessed for mRNA expression of regulatory molecules, such as IL-10 or FOXP3, directly *ex vivo* or after *in vitro* stimulation. (B) Flow cytometry can be used to assess expression of up to 17 different cell markers simultaneously. Cells from whole blood and PBMC can be stained and analyzed. The most common definition of Tregs is the population expressing CD4, FOXP3 and high levels of CD25; a representative example of Treg gating within $CD4^+$ T cells is shown (B-i). Furthermore, when selecting these cells, the expression of several other molecules can be analyzed as depicted in B-ii, where examples are shown of Helios and CD127 expression, two markers used for further characterization of Tregs. In C and D, options for functional characterization of Tregs are depicted. (C) Isolation of Tregs can be performed using magnetic beads (C-i) or fluorescent activated cell sorting (C-ii). The acquired cells can be directly co-cultured with other cell populations (C-iii) to determine the inhibitory capacity of Tregs, alternatively the cells can be expanded *in vitro* (C-iv) to obtain sufficient cell numbers for further applications, such as immunotherapy. (D) A more indirect, but more field applicable approach for the functional analysis of Tregs is the depletion method. By using magnetic bead isolation of CD25-positive or -highly positive cells (D-i), the flow-through can be regarded as Treg-depleted cells (D-ii), whereas the isolated Tregs can be added back to the flow-through to create a "mock"-depleted cell fraction (D-iii). Both cell populations can furthermore be cultured *in vitro* and the difference in immune responses can be analyzed.

– Representation adapted from Miltenyi Biotec –

Challenges in immunoepidemiological field studies

The immune regulatory network is thought to be essential for helminth-induced modulation of parasite-specific as well as bystander responses. Many research groups in affluent countries have addressed the question if and how helminths affect the immune system and whether, through modulation of bystander responses, helminths could influence the outcome of vaccinations or inflammatory diseases. Whereas the work has mainly been conducted in animal models, travellers or experimentally infected humans, only few groups have taken these questions to areas where helminth infections are highly endemic. Field studies in remote areas are complicated by the logistic challenges, lack of advanced technologies and, possibly, cultural obstacles. However, these studies analyzing human samples are of utmost importance for understanding the real-life situation, and moreover, for the opportunities of health education and bilateral knowledge transfer. Despite the difficulties, in the last decades several investigators have established collaborations with scientists in low-resource settings and these have generated important insight into the interaction of helminths with the immune system, vaccines, other infections and allergies.

It was established in various study sites that cellular immune responses to tetanus[62-64], cholera[65], BCG[66,67] and influenza[68] vaccines are impaired in helminth-infected individuals and some studies have shown increased responses to vaccines after anthelmintic treatment[62,67,69], although many were not placebo-controlled. The effect of helminths on coinfections has been addressed in a number of studies, but mostly in a cross-sectional manner. Helminth and malarial infections have overlapping distributions in tropical regions, raising the question what impact helminth infections may have on the plasmodial parasites that cause malaria. There is much controversy surrounding the effect of helminth infections on malarial parasitemia and clinical malaria episodes. Most studies have used cross-sectional designs and have variously reported detrimental[70,71] or beneficial[72,73] or no[74,75] effect of helminths on either burden of infection or clinical outcomes. Studies of anthelmintic treatment are expected to be more informative, but the trials that have been conducted so far have also shown detrimental[76] or beneficial[77] effects in small groups of children. The relationship between helminth infections and allergy has received much attention, also in terms of clinical trials conducted in areas endemic for helminth infections. Although the majority of cross-sectional studies have reported inverse associations between helminth infections and skin prick test (SPT) reactivity[78], a number show that certain helminths may increase the risk of atopy[79,80]. Two randomized trials with albendazole treatment have been carried out in cohorts of school children. A study in Ecuador showed no change in either SPT reactivity to allergens or allergic symptoms, but this study did not include a placebo group[81], while in a trial in

Vietnam, one year of albendazole treatment increased SPT reactivity but also did not change clinical allergy to any significant degree[82]. It has been suggested that longer anthelmintic treatment might be needed to reveal the modulatory effect of helminths[83].

So far, most field studies have not assessed immune alterations in parallel to clinical consequences of helminth elimination. The two albendazole trials assessing the effect on allergy in school children demonstrated lower IL-10 production in response to helminth antigens after anthelmintic treatment[82,84], suggesting a role for parasite-specific IL-10. The Ecuador study also showed an increase in helminth-specific Th2 responses[84] and enhanced Th2 responses were furthermore seen in a mebendazole trial in infants from Pemba[85] and after praziquantel treatment of pregnant women in Uganda[86], but two of these studies were not placebo-controlled and none of them involved a whole community but focused on specific age groups. There is therefore a need for randomized controlled interventional studies assessing the effect of deworming on the prevalence of coinfections and allergy, together with detailed assessment of immunological parameters, which might help us understand the causal pathways.

Scope and aims of this thesis

Since parasitic infections are still highly prevalent in tropical areas, there are opportunities to study the underlying immunological processes that might explain the possible beneficial effects of helminth infections. In particular, areas with few or no history of mass drug administration would be suited to analyze the 'natural' situation in which humans live with worms. By looking into our past, we may be able to find solutions for the current struggle worldwide with immune-associated diseases, not only in affluent countries but also in urban centers of the less affluent regions of the world. It is important to note that such studies are expected to help anticipate the consequences of the future epidemiological transition for low-to middle-income countries and thus prepare the health care systems for the challenge facing them. The dilemma between deworming and helminth immunotherapy is pressing and of major global public health impact.
The regulatory network, where Tregs play an important role, is thought to be central to the relationship of parasites with coinfections and inflammatory diseases. The characterization of this regulatory network, and helminth-induced Tregs in particular, forms the focus of this thesis. The specific aims are as follows:

i. To characterize T cell responses during parasitic infections
ii. To explore the mechanisms of immune modulation employed by parasites
    a. Proportions and phenotype of Tregs during parasitic infection
    b. Treg suppressive capacity measured by establishing a field-applicable assay
iii. To assess the immunological consequences of deworming
iv. To assess the clinical outcomes of deworming, in terms of malaria and allergy

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